REMARKS

The Final Office Action mailed May 28, 2004, has been carefully reviewed and the following comments are made in response thereto.

Claims 27, 39, 43 and 76-93 are currently pending. Claims 27 and 76-92 are withdrawn as being directed to non-elected subject matter. Therefore, independent claim 39 and dependent claims 43 and 93 are currently before the Examiner.

In view of the following remarks, Applicants respectfully request reconsideration and reexamination of this application and the timely allowance of all of the pending claims.

Restriction Requirement

The Office Action states that "claim 92 is withdrawn from consideration as being directed to a nonelected invention," since "the originally examined invention is drawn to a DNA sequence encoding a repressor protein under the control of an inducible promoter, whereas claim 92 is drawn to a DNA sequence encoding an inhibitor under the control of an inducible promoter" (page 2, paragraph 5).

In response to the Restriction Requirement mailed 25 February 2003, Applicants elected Group I ("an expression system comprising recombinases") and species (A) ("FLP recombinase"), (ii) ("a gene encoding an inhibitor"), (cc) ("where the gene is under the control of inducible promoter of a AlcA gene"). This election was communicated to the U.S. PTO on 24 June 2003.

The Office Action mailed on 2 October 2003, rejected independent claim 39 as being ambiguous allegedly because it included "components (a)+ [(b)i or (b)ii] and (c) and (d)". In response, Applicants amended claim 39 by deleting step (b)(ii) directed to "a gene encoding an inhibitor of the recombinase" and amending step b(i) so that it was directed to "a DNA sequence encoding a repressor protein". The amended claim 39 was subsequently examined by the Examiner in the Final Office Action mailed on 28 May 2004. Therefore, the Examiner is requested to acknowledge on the record that the species election has been effectively shifted to b(i), a repressor, from b(ii), an inhibitor. If so, then Applicants acknowledge the Examiner's statement that claim 92 has been withdrawn as being drawn to a non-elected invention.

Rejection Under 35 U.S.C. § 112, First Paragraph

Claims 39, 43 and 93 are rejected under 35 U.S.C. § 112, first paragraph, purportedly for failing to comply with the enablement requirement. Apparently, the Examiner does not believe that the claimed FLP recombinase expression system would operate in whole plants according to the methods disclosed in the application. In briefly discussing the evidence of enablement provided by Applicants, the Office Actions states that the Lyznik *et al.* article referenced in the specification "uses plant cells and protoplasts, which are different in complexity with whole plants".

Example 4 was previously cited by Applicants as support for the as-claimed invention. This Example provides an example of FLP mediated excision of a FRT-flanked PAT gene and suppression of effect by an inducible repressor gene using transgenic tobacco plants as a representative plant model. The Office Action fails to explain why Example 4 does not enable the invention as presently claimed. An applicant need not have actually reduced the invention to practice prior to filing. *Gould v. Quigg*, 822 F.2d 1074, 1078, 3 USPQ 2d 1302, 1304 (Fed. Cir. 1987). Furthermore, "a single working example in the specification for a claimed invention is enough to preclude a rejection which states that nothing is enabled since at least that embodiment would be enabled." See, M.P.E.P. § 2164.02.

The Office Action argues that since the experiments in Lyznik *et al.* were preformed on plant protoplasts of maize and rice, this does not enable the FLP recombinase system in whole plants. Applicants strongly disagree. As is well known to one skilled in the transgenic plant arts, a plant protoplast is simply a plant cell from which the cell wall has been removed; the rest of the cell, including the cell membrane, remains intact. The internal cellular components and 'machinery' in protoplasts are the same as those in cells of whole plants of the same species. Therefore, protoplasts are frequently used predictively to test the functionality of recombinant DNA constructs *in planta* as an alternative to using whole plants. Applicants believe, therefore, that the functionality of the FLP system in maize and rice protoplasts as demonstrated in Lyznik *et al.* is a clear indication that the FLP recombinase system of *Saccharomyces cerevisiae* is functional in plant cells.

However, if the Examiner requires even further evidence that the claimed invention operates as described in the specification, such evidence is readily available. See, for example,

the attached representative articles by Lloyd *et al.* (Mol. Gen. Genet. (1994) 242(6):653-657); Sonti *et al.* (Plant Mol. Biol. (1995) 28(6):1127-1132); and Kilby *et al.* (Plant J. (1995) 8(5):637-652), as well as U.S. Patent No. 5,527,695, each of which support the enablement of the currently claimed invention. Copies of each of these references are attached as Exhibits A-D.

Lloyd *et al.* (Exhibit A) teach that "the FLP/FRT site-specific recombination system of *Saccharomyces cerevisiae* was expressed in stably transformed tobacco plants" (Abstract). Tobacco is the representative plant utilized in Example 4 of the present application.

Sonti *et al.* (Exhibit B) teach the introduction of the yeast FLP recombinase system into *Arabidopsis* plants and that "FLP-dependent Gus activity was observed in both transient assays and transgenic plants" (Abstract).

Kilby *et al.* (Exhibit C) teach that "FLP site-specific recombinase was expressed in stably transformed tobacco and Arabidopsis" (Abstract). Again, tobacco is the representative plant utilized in Example 4 of the present invention.

U.S. Patent No. 5,527,695, filed in January 1993 and listing Leszek A. Lyznik as one of the inventors (Exhibit D), discloses methods of producing fertile, transgenic plants that include FLP/FRT recombinase constructs.

Thus, contrary to the assertions of the Office Action, ample evidence is available that the FLP/FRT recombinase system utilized in the present invention would operate in whole plants as described in Example 4. Clearly, Example 4 enables the invention as claimed.

The present continuation application has been pending since September 27, 1999. For the above stated reasons, the Examiner is respectfully requested to withdraw the only outstanding rejection and allow claims 39, 43 and 93.

Conclusion

In view of the foregoing remarks, Applicants respectfully request withdrawal of all outstanding rejections and early notice of allowance to that effect. Should the Examiner believe that a telephonic interview would expedite prosecution and allowance of this application, he/she is encouraged to contact the undersigned at her convenience.

Except for issue fees payable under 37 C.F.R. § 1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. §§ 1.16 and 1.17 which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account No. 50-0310. This paragraph is intended to be a **CONSTRUCTIVE PETITION FOR EXTENSION OF TIME** in accordance with 37 C.F.R. § 1.136(a)(3).

Respectfully submitted,

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Functional expression of the yeast FLP/FRT site-specific recombination system in *Nicotiana tabacum*

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Abstract. The FLP/FRT site-specific recombination system of *Saccharomyces cerevisiae* was expressed in stably transformed tobacco plants. The FLP protein efficiently catalyzes recombination between two directly repeated *F*LP *r*ecombination *t*arget (FRT) sites, deleting the sequence between them. In the constructs tested here, this deletion places the CaMV 35S promoter adjacent to a hygromycin resistance marker; transcriptional activation of the marker allows direct selection of recombination events. After crossing plants containing an integrated FLP expression construct with plants containing a FLP substrate, F_1 seedlings can be selected directly for hygromycin resistance, indicating that recombination occurs at, or very early after zygote formation. Molecular analysis confirmed the expected recombination product.

Key words: FLP/FRT – Site-specific recombination – Tobacco

Introduction

The yeast 2 μ plasmid encodes a site-specific recombination system termed FLP/FRT (for review see Cox 1988). FLP/FRT is thought to be involved in regulating 2 μ plasmid copy number (Futcher 1986). The recombination site, termed FRT for *F*LP *r*ecombination *t*arget comprises 34 bp. Each FRT includes two 13 bp inverted repeats separated by an asymmetric 8 bp spacer. The FRT site is the substrate of a 48 kDa protein termed FLP. The FLP recombinase has been shown to catalyze intra- and intermolecular recombination events between two FRT sites in vivo and in vitro, without regard to DNA topology (Cox 1988). This system has been successfully expressed in several heterologous species including *Escherichia coli* (Cox 1983), *Drosophila melanogaster* (Golic and Lindquist 1989), and mammalian cells (O'Gorman et al. 1991).

In plant genetics and molecular biology, site-specific recombination is a potentially valuable tool for directing deletions, inversions, translocations, and site-specific integrations in the plant genome. Expression of the prokaryotic *loxP*-Cre site-specific recombination system has recently been reported in plant cells (Dale and Ow 1990; Odell et al. 1990; Dale and Ow 1991; Bayley et al. 1992; Russell et al. 1992). It was reasoned that a eukaryotic site-specific recombinase that has evolved to cross the nuclear membrane and function on nuclear recombination sites, such as the FLP recombinase, might be more efficient in plants. It is also conceivable that one would want to express two different site-specific recombination systems simultaneously or differentially in the same plant for specific recombinational goals.

As a test case for FLP/FRT function in plants, a binary system was employed whereby FLP was expressed in one transgenic line and the target was integrated in another. Crossing these two lines brings FLP and FLP substrate together in the same cell, a precondition for the site-specific recombination event (Fig. 1). The question addressed here was whether FLP could mediate a site-specific deletion event to bring together a promoter and selectable marker, thereby transcriptionally activating the marker. This work demonstrates that the FLP recombinase can efficiently mediate site-specific deletion events on substrates stably integrated in the genome of tobacco after genetic hybridization of the FLP and substrate lines.

Materials and methods

Molecular techniques. All plasmid and DNA manipulations were performed according to standard techniques (Sambrook et al. 1989).

The FLP expression vector was constructed as follows. pMCC4 (Cox 1983) was digested with *Sph*I and *Pvu*II. The 2.8 kb FLP-containing fragment was cloned

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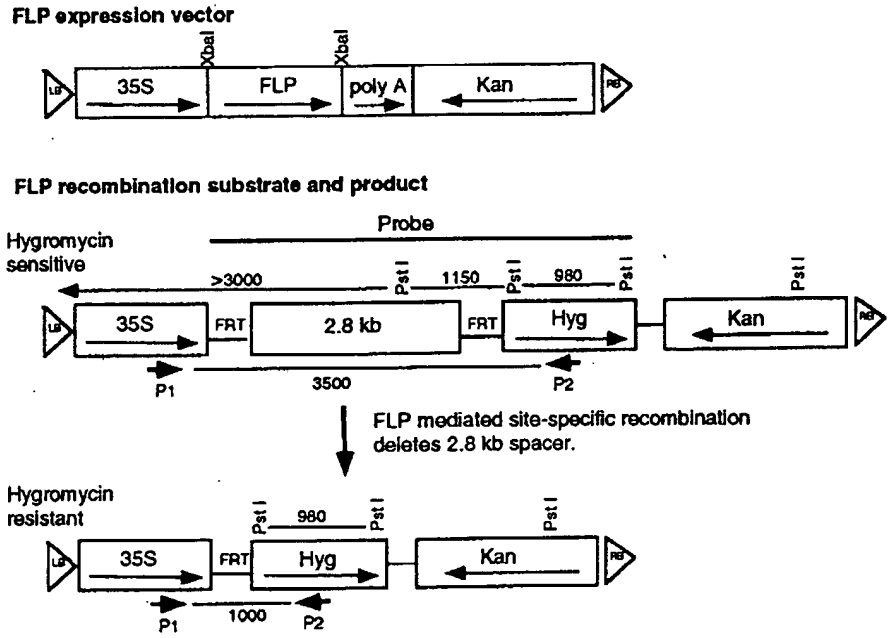

Fig. 1. Schematic diagrams of the FLP expression vector and the FLP substrate. The substrate is shown as it is integrated in the plant genome, before and after site-specific recombination. Before recombination, the plants are hygromycin sensitive, after recombination the plants are hygromycin resistant. The extent of the probe used in the DNA blot in Fig. 3 is shown above the unrecombined substrate. The sizes of the hybridizing *Pst*I restriction fragments are shown above the unrecombined and recombined substrates. The positions of the PCR primers used in Fig. 3 and the sizes of the potential PCR products are shown below the substrates

into pIC20R (Marsh et al. 1984), prepared by digestion with *Sph*I and *Eco*RV, to create pAL48. pAL48 was cut with *Xba*I, and the 1.5 kb FLP-containing fragment was cloned into pKYLX71 (Schardl et al. 1987; Lloyd et al. 1992) prepared by digestion with *Xba*I and treatment with calf intestinal alkaline phosphatase (CIP). A resulting plasmid containing the FLP coding region in the correct orientation was named pAL53. pAL53 contains the FLP coding region under the transcriptional control of the CaMV 35S promoter (Fig. 1).

A schematic diagram of the FLP substrate integrated in the plant genome is shown in Fig. 1. The FLP substrate vector was constructed as follows. pKYLX71 was digested with *Eco*RI and *Cla*I and the large vector fragment was retained. pIC20R was digested with *Eco*RI and *Cla*I and ligated directly to the large pKYLX71 fragment. Resulting plasmids were screened for one that acquired the 75 bp region of the pIC20R polylinker to create pAL47. pAL47 contains the pIC20R polylinker and a kanamycin resistance, plant selectable marker between the T-DNA left and right borders. pCaMVHyg was created by replacing the *Bam*HI nptII fragment in pCaMVNeo (Fromm et al. 1986) with a 1.3 kb *Bam*HI–*Bgl*II fragment containing the *E. coli hph* coding region (M. Fromm and V. Walbot, personal communication). This destroys both sites of the *Bgl*II *Bam*HI fusion at the 3′ end of the *hph* gene. pCaMVHyg was digested with *Hin*dIII and *Bam*HI and the 1.5 kb fragment comprising the *hph* coding region and nos polyA addition site was isolated. This fragment was ligated to the large fragment of *Hin*dIII + *Bam*HI-digested pBA112 (Andrews et al. 1985) to create pAL49. pAL49 contains a FRT at the 3′ end of the *hph* gene. pKYLX71 was digested with *Hin*dIII and *Eco*RI and the 900 bp CaMV 35S promoter fragment was isolated. pJFS122 (Senecoff et al. 1985) was digested with *Hin*dIII and *Bam*HI and treated with CIP. This releases the FRT site contained on pJFS122. pBSKS+ (Stratagene) was digested with *Hin*dIII and *Bam*HI and the large vector fragment was isolated. A three-way ligation was performed between the above pKYLX71, pJFS122, and pBSKS+ fragments. Resulting plasmids were screened for one with the CaMV 35S promoter from pKYLX71 oriented to read towards the FRT site of pJFS122, both inserted into pBSKS+ to obtain pAL50. pAL50 was partially digested with *Hin*dIII, linear full-length molecules were isolated, the overhangs were filled in with the Klenow fragment, and the plasmid was reclosed. Resulting plasmids were screened for loss of the *Hin*dIII site between the CaMV 35S promoter and the FRT site to isolate pAL51. The FRT site contains an *Xba*I restriction site asymmetrically placed in the 8 bp internal spacer region. Ligation of *Xba*I-digested FRT sites will recreate a functional FRT site in one orientation only. pAL57 was created by a three-way ligation between the following DNA fragments: the large vector fragment of pAL47 digested with *Eco*RI and *Hin*dIII and treated with CIP; the small *hph*/FRT fragment of pAL49 digested with *Hin*dIII and *Xba*I; and the small CaMV/FRT fragment of pAL51 digested with *Xba*I and *Eco*RI. This ligation recreates a single FRT site located between the CaMV 35S promoter and the *hph* gene. pAL57 contains the CaMV 35S–FRT-*hph* sequences and a kanamycin resistance, plant selectable marker between the *Agrobacterium* T-DNA right and left borders. pAL57 was digested with *Cla*I and *Bgl*II, the overhangs were filled in with the Klenow fragment and sealed to create pAL77. This removes an *Xba*I site and other polylinker sites outside the FRT sequence. pAL77 was digested with *Xba*I, treated with CIP and ligated to *Xba*I-cut pJFS36sym (Senecoff et al. 1985). pJFS36sym is an ampicillin-resistant, tetracycline-sensitive pBR322 derivative containing a single FRT site. Tetracycline- (due to pKYLX71) and ampicillin-resistant *E. coli* transformants were screened for a plasmid with the desired insert

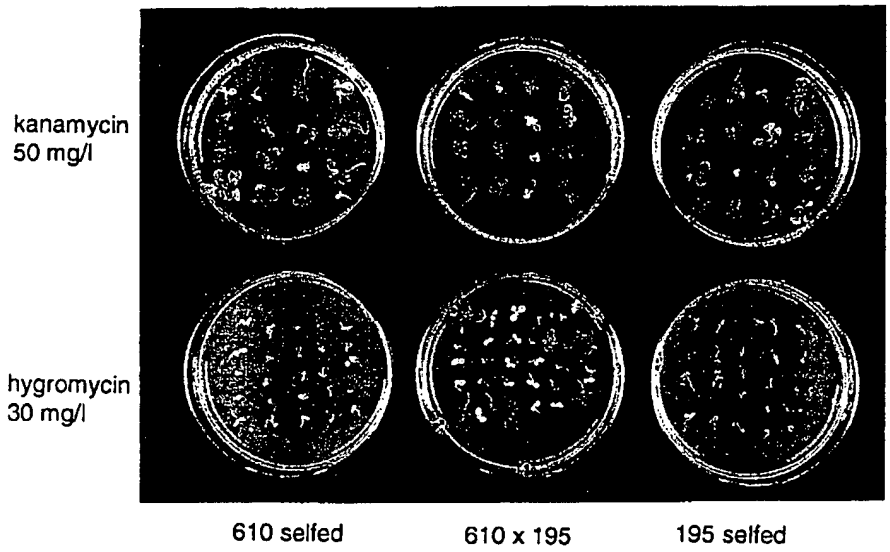

Fig. 2. Analysis of antibiotic resistance of selfed and hybrid progeny of two transformants: 195, FLP expression and 610, FLP substrate. The *upper row* seeds were plated on medium containing 50 mg/l kanamycin, *lower row* seeds were plated on 30 mg/l hygromycin. On the left are progeny of selfed plant 610, on the right are progeny of selfed plant 195, in the center are F_1 hybrid progeny of 610 × 195

Table 1. Antibiotic resistance segregation data for progeny of selfed and crossed transformants

Plant or cross	Kanamycin resistant	Kanamycin sensitive	Hygromycin resistant	Hygromycin sensitive
195 selfed	26	6	0	34
673 selfed	24	6	0	48
610 selfed	12	4	0	25
606 selfed	33	8	0	35
610 × 195	11	5	28	132
610 × 673	38	9	23	98
606 × 195	23	5	7	31
606 × 673	21	6	4	28

Plants 195 and 673 contain pAL53, the FLP expression construct. Plants 610 and 606 contain pAL78, the FLP substrate construct.
Numbers are based on the seedling growth assay shown in Fig. 2

orientation to obtain pAL78. pJFS36sym and pAL77 each contain one FRT site containing a unique *Xba*I restriction site. By ligating these two together, the CaMV 35S promoter is separated from the hygromycin resistance coding region by approximately 2.8 kb of plasmid sequence. In the desired orientation, two directly repeated functional FRT sites are recreated (Fig. 1).

In the R.W. Davis lab collection: pAL48 = pNN491; pAL53 = pNN492; pAL47 = pNN493; pAL49 = pNN494; pAL50 = pNN495; pAL51 = 496; pAL57 = pNN497; pAL77 = pNN498; pAL78 = pNN499.

Plant manipulations. Leaf pieces of greenhouse-grown *Nicotiana tabacum* cv. *Xanthi* were transformed essentially as described by Horsch et al. (1985). The *Agrobacterium tumefaciens* strain used was GV3101 containing the helper plasmid pMP90 (Koncz and Schell 1986). To verify the existence of functional kanamycin or hygromycin resistance markers in hybrid or selfed progeny of tobacco transformants, surface-sterilized seed was plated on MS medium (Sigma) containing 1% sucrose and either 50 mg/l kanamycin or 30 mg/l hygromycin as appropriate. Green healthy seedlings were scored resistant. Bleached seedlings whose development was arrested were scored as sensitive.

DNA blots. Plant DNA was isolated according to Walbot and Warren (1988). Digested DNA fragments were separated by gel electrophoresis and capillary transferred to Nytran membrane (Schleicher and Schuell). Transfer, prehybridization, hybridization, and washing was done according to the manufacturer's recommendations. The probe was radiolabeled pAL49, which is essentially pBR322 containing the hygromycin resistance marker. The probe was generated using a Prime-It kit (Stratagene) according to the manufacturer's recommendations.

Polymerase chain reaction (PCR). DNA isolated from tobacco plants was subjected to the PCR using Hot Tub polymerase (Amersham) and the buffer supplied by the vendor. An Ericomp thermocycler was used with the following parameters: 94° C for 4 min; 25 cycles of 94° C for 30 sec, 55° C for 30 sec, and 72° C for 45 sec; 72° C for 7 min. Primers used were as follows: P1 is specific for the CaMV 35S promoter, 5′-GTGGATTGATGTGATATCTC-3′; P2 is specific for the *hph* gene, 5′-

CGCACTGACGGTGTCGTC-3′. The positions and orientations of P1 and P2 are shown in Fig. 1. PCR products are shown in Fig. 3.

Results

The *E. coli hph* gene has been shown to confer hygromycin resistance on plant cells when placed under the control of appropriate plant regulatory signals (Waldron et al. 1985; van den Elzen et al. 1985; Lloyd et al. 1986). pAL78, the unrecombined FLP substrate with an interrupted *hph* gene (Fig. 1), when transformed into tobacco gave very high level of resistance to kanamycin but no resistance to hygromycin above untransformed background levels, both during initial transformation experiments (data not shown) and in progeny of selfed transformants subsequently obtained (Fig. 2; Table 1). A plasmid containing only a single FRT site between the promoter and *hph* gene, pAL57 (Materials and methods), gave very high levels of resistance to both kanamycin and hygromycin (data not shown). These data confirmed that the presence of 2.8 kb of plasmid sequence between the CaMV 35S promoter and the *hph* structural gene was sufficient to eliminate functional expression of *hph* and that the presence of a single FRT site did not interfere with this expression.

Primary transformants were selfed or crossed to obtain progeny for analysis. Table 1 and Fig. 2 show the results of crossing some representative transformants. Plant 610 was transformed with pAL78, the FLP substrate construct. Plant 195 was transformed with pAL53, the FLP expression construct. Selfed progeny of both plants show approximately a 3 to 1 segregation for kanamycin resistance, indicating insertion of the T-DNA at a single locus. Selfed progeny of both plants show no resistance to hygromycin. Crossing plants 610 and 195 combines the FLP expression and substrate constructs together in the same cell. Because both of these plants are segregating for a single T-DNA locus, only 1/4 of the hybrid progeny would be expected to receive both the FLP expression locus and the substrate locus. Fig. 2 illustrates the seedling growth assay to determine antibiotic resistance and clearly shows that hygromycin resistance is restricted to the FLP/FRT hybrids. Table 1 shows that 17.5% of the progeny in these crosses are hygromycin resistant. Thus these progeny can now functionally express the hygromycin resistance marker. This hygromycin resistance is strictly dependent on crossing the FLP expression and FLP substrate plants. Table 1 shows that crosses involving these and other transformants give essentially the same results. The fact that none of these crosses gave 25% hygromycin-resistant progeny may reflect the level of inefficiency of this recombination system.

Molecular analysis of the T-DNA at the substrate locus indicates that site-specific excision of the sequence between the directly repeated FRT sites has occurred in the hygromycin-resistant progeny, as diagrammed in Fig. 1. Polymerase chain reaction (PCR) analysis of progeny of the selfed parents and hygromycin-resistant hybrid progeny, using primers P1 and P2, revealed the

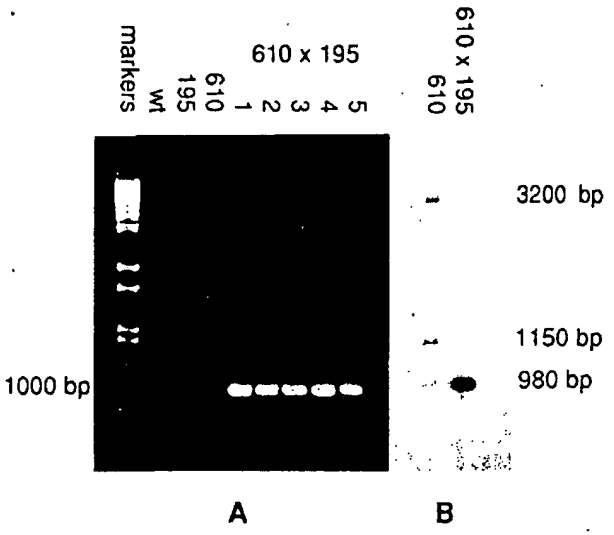

Fig. 3A, B DNA analysis of selfed and hybrid progeny of transformants: 195, FLP expression and 610, FLP substrate. A PCR analysis. Lanes are as marked. Primers used were P1 and P2, described in the text and shown in Fig 1. 610 × 195 hybrid progeny 1–5 were hygromycin resistant. All others were hygromycin sensitive. Marker lanes contain λ DNA digested with *Bst*EII B DNA blot analysis. Lane 610 is the same DNA used in the PCR analysis. Lane 610 × 195 is pooled DNA from the 5 hybrid progeny used in the PCR analysis. The probe used is described in the text and shown in the schematic diagram in Fig. 1

presence of the 1000 bp CaMV 35S/FRT/hyg recombination product in the hygromycin-resistant hybrid progeny only, not in progeny of either selfed parent (Fig. 3A). This new junction has lost the 2.8 kb of intervening plasmid sequence. Although the unmodified substrate plant could theoretically give a 3.5 kb PCR product with the primers used, it is not seen under the conditions used here.

DNA blot analysis of the substrate locus before and after FLP-mediated recombinations shows the loss of the intervening plasmid sequence in the hygromycin-resistant hybrid progeny. Pooled DNA samples from several kanamycin-resistant progeny of the selfed substrate plant, 610, and 5 hygromycin-resistant 195 × 610 hybrids were digested with *Pst*I and the restriction fragments were separated by agarose gel electrophoresis. The gel was blotted and probed with radiolabeled pAL49. pAL49 is essentially pBR322 containing the hygromycin resistance marker. This probe will hybridize to the intervening plasmid in the substrate construct as well as the *hph* sequences, as diagrammed in Fig. 1. Fig. 3B shows the expected 1150 bp intervening plasmid sequences, a border fragment of approximately 3200 bp, as well as the 980 bp *hph* sequence in the unrecombined substrate plant. The sample from the hygromycin-resistant hybrid progeny retains the 980 bp *hph* sequence, but has lost all detectable intervening plasmid sequence and the remaining border fragment no longer hybridizes to this probe. The 610 × 195 lane was overloaded to try to detect any unrecombined substrate or circular recombination product remaining in these plants. Neither was detected.

Discussion

The *S. cerevisae* FLP/FRT site-specific recombination system has been reported to direct several types of recombination events in heterologous species. In *E. coli* it has been used to invert sequences between inverted FRT sites on a plasmid (Cox 1983) as well as to integrate foreign DNA containing an FRT site at a chromosomally located site (Huang et al. 1991). In *Drosophila*, FLP/FRT has been used to delete sequences between directly repeated FRT sites (Golic and Lindquist 1989). In some cases this deleted circular DNA must have reintegrated via FLP/FRT on a sister chromatid, leading to amplified tandem copies of the sequence. FLP/FRT has also been used to direct recombination between non-sister chromatids of homologous chromosomes in *Drosophila* (Golic 1991). In mammalian cells, FLP/FRT has been used to excise sequences between chromosomally located FRT sites and integrate foreign circular FRT-containing DNA at a chromosomally located FRT site (O'Gorman et al. 1991).

This study demonstrates that the *S. cerevisiae* FLP/FRT site-specific recombination system functions in tobacco plants. The system employed here deletes a plasmid sequence inserted between two directly repeated FRT sites to place the CaMV 35S promoter adjacent to a hygromycin resistance marker. This recombination event transcriptionally activates the hygromycin resistance marker, leaving a single FRT site in the *hph* mRNA leader, and can be directly selected in hybrid progeny of FLP expression and substrate plants. The recombination event occurs very efficiently and soon after zygote formation. This extends the functional range of activity of this system from yeast, *E. coli*, *Drosophila*, and mammalian cells to plants. It is interesting to note that attempts to induce FLP/FRT recombination in the plant *Arabidopsis thaliana*, using the constructs described in this study, have so far failed for unknown reasons.

The recombination scheme described in this paper is analogous to previously published reports of *loxP*/Cre-mediated site-specific deletions in tobacco and *Arabidopsis* (Dale and Ow 1991; Russell et al. 1992; Bayley et al. 1992). Further characterization will be needed to compare the efficiencies of these two systems directly. FLP/FRT should provide an alternative and complementary method to the *loxP*-Cre site-specific recombination system for use in tobacco and perhaps other plant species.

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Plant Molecular Biology 28: 1127–1132, 1995.

Short communication

Activity of the yeast FLP recombinase in *Arabidopsis*

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Abstract

The coding sequence for FLP recombinase, originally from the 2 μ plasmid of *Saccharomyces cerevisiae*, was introduced into *Arabidopsis* behind the cauliflower mosaic virus 35S promoter. FLP activity was monitored by the glucuronidase activity resulting from inversion of an antisense-oriented *GUS* reporter gene flanked by a pair of *FRT* target sites in inverted repeat. FLP-dependent Gus activity was observed in both transient assays and transgenic plants. The FLP system will be useful for a variety of *in planta* genetic manipulations.

Site-specific recombinases (review [27]) generally promote reciprocal crossing-over specifically between a pair of target nucleotide sequences. As a result the stretch of DNA lying between the two sites is deleted when the two are in direct repeat, and inverted when the two are in inverted repeat.

Site-specific eukaryotic and prokaryotic recombinases used for engineering of heterologous genomes *in vivo* in various organisms include *Saccharomyces cerevisiae* FLP recombinase in *Pichia pastoris* [6], *Drosophila* [9, 10] and mammalian cells [22]; a similar enzyme from *Zygosaccharomyces rouxii* in *S. cerevisiae* [20]; Cre recombinase from phage P1 in *S. cerevisiae* [28] and mouse [29]; and *Drosophila* P-element transposase in *Drosophila* [4]. For plants, Cre has been used extensively in tobacco [3, 7, 8, 21, 25, 26]. In addition, R recombinase of *Z. rouxii* has been shown to function in tobacco protoplasts [23], and an allele of phage Mu Gin recombinase although lethal to function in *Arabidopsis* and tobacco protoplasts [18].

A multiplicity of site-specific recombinases will clearly allow a corresponding increase in the sophistication of *in planta* genetic manipulation. We have been studying activity of the yeast 2 μ plasmid FLP recombinase (review [5]), which although eukaryotic rather than prokaryotic is mechanistically similar to Cre and Gin (see [27]). Each of its *FRT* target sites is based on two 13 bp repeats flanking an 8 bp asymmetric core, which the enzyme cuts to give an 8 base 5′ overhang via a phosphotyrosyl-bound intermediate [1, 13, 30]. Thus FLP will promote either deletion or inversion depending on whether the cores are oriented parallel or anti-parallel, and likewise, when the *FRT* sites are on different chromosomes, translocation.

Here we report that FLP, introduced into *Arabidopsis* together with *FRT* sites, promotes the expected rearrangement, in this case, inversion, both in transient assays with co-cultivated root explants and also in transgenic plants. While this work was in progress, we learned that inversion by FLP has also been shown in transient assays with transfected maize protoplasts [16, 17], and deletion by FLP in transgenic tobacco although activity in transgenic *Arabidopsis* was not detected [15].

The *FLP*-coding sequence is the 1.45 kb *Sph* I-*Bam* HI fragment from plasmid pUC19-FLP, and the *FRT* site is the 89 bp *Eco* RI-*Hin*d III fragment from plasmid pUC19-J3 [11]. Constructs made with the cauliflower mosaic virus 35S promoter (P35S), *Escherichia coli GUS* (from plasmid pRAJ270) and 3′ nos terminator by standard procedures (Fig. 1) were cloned into the T-DNA binary vector pGA-3-Sh [24] or its derivatives in place of the *Eco* RI-*Sst* I fragment that includes the *Sh* gene (pGA-FLP, pAT4−, pAT4+) or into the *Sca* I site (pRS1.2). Plasmids were mobilized into *Agrobacterium* strain AGL1 [14] by triparental mating with *E. coli* (pRK600) as helper and selection for resistance to carbenicillin (Sigma; 100 μg/ml). Plants were grown on germination medium (containing MS salts, agarose 0.8%, sucrose 3%, Gamborg's vitamin solution 2 mg/l) in a Percival growth chamber at 20 °C with a 16 h photoperiod. Seeds were sterillized in 70% ethanol for 2 min, followed by Clorox for 15 min, followed by 5 to 6 washes in sterile H_2O. For root explants, seeds were germinated on agarose plates oriented vertically so that roots grew along the agar surface. Transformation was according to Marton and Browse [19]: roots from plants 10 to 14 days old were cultured for 4 days on callus-inducing medium, cut into 1 cm explants while submerged in a saturated culture of *Agrobacterium*, blotted dry, and incubated on callus-inducing medium for 2 days, followed by 2 more days on callus-inducing medium plus antibiotic (30 parts ticarcillin [Sigma] to 1 part potassium clavulanate [SmithKline Beecham]; 50 μg/ml) to prevent bacterial overgrowth. For transient assays, explants were then stained directly for Gus activity. For transformation, explants were then

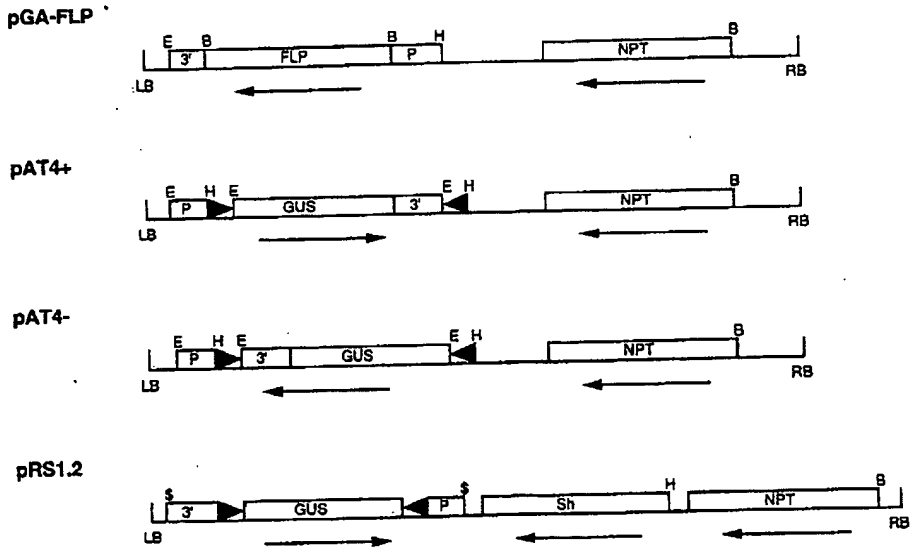

Fig. 1. Chimeric T-DNA constructs. *FLP*, FLP recombinase coding sequence; filled triangle, *FRT* target site; GUS, β-glucuronidase; *NPT*, neomycin phosphotransferase; *Sh*, bleomycin resistance of *Streptoalloteichus hindustanicus*; P, cauliflower mosaic virus 35S promoter; 3′, nos3′ terminator; LB, RB, left and right T-DNA border sequences; B, *Bam* HI; H, *Hin*d III; E, *Eco* RI; $, *Sca* I destroyed in cloning. Arrows show sense orientation of coding sequence, not transcription. Distances are not to scale.

transferred to shoot-including medium containing kanamycin (plant-tested, Sigma; 25 μg/ml) or phleomycin (Sigma; 20 μg/ml), and subsequently to soil for seed set. The pGA-FLP line used here contains a single copy of the insert as determined by Southern analysis (not shown), and is homozygous by segregation analysis. The pAT4 – line originally contained two independently segregating insert loci; a derivative subline containing a single locus was isolated and is used here. Tissue obtained from plants grown axenically was incubated at 37 °C in 50 mM sodium phosphate buffer pH 7.0 containing XGlc (1 mg/ml). Root explants were stained for 12 to 24 h, and leaves for 24 to 36 h. After staining, tissue was cleared in 95% ethanol at 65 °C for 6 h to remove chlorophyll and carotenoids. Positive controls were plants carrying pAT4 + (Fig. 1).

We tested activity in a two-component system, with FLP produced from pGA – FLP, carrying the *FLP* gene driven by P35S, acting on a pair of *FRT* target sites in a second T-DNA reporter construct, pAT4 – carrying antisense *GUS* bracketed by two *FRT* in inverted repeat (Fig. 1). FLP activity will invert *GUS* from antisense to sense, resulting in a corresponding change in phenotype from Gus^- to Gus^+, which can be detected by staining with the chromogenic substrate XGlc. The similar construct pAT4 +, with *GUS* gene bracketed by inverted repeat *FRT* in the sense orientation, serves as control. In some experiments we used instead the antisense reporter pRS1.2, which carries an additional selectable drug marker, and has the nos3′ terminator outside rather than inside the invertable region (Fig. 1).

In a transient assay, Janssen and Garner [12], using *Arabidopsis* leaf explants co-cultivated with *Agrobacterium*, found that a *GUS* gene transferred to plant cells showed a high level of expression that appeared independent of integration into the genome. We used a similar design with root explants to test transfer either of a *FLP* expression construct into a plant already carrying an *FRT* target reporter construct or, conversely, of an *FRT* construct into a plant already carrying a *FLP* construct. We first isolated derivatives of wild-type (ecotype RLD) stably transformed with either the P35S-*FLP* expression construct pGA-FLP, or the *FRT* reporter antisense *GUS* construct pAT4 – (Fig. 1). Roots of the pGA-FLP line were then co-cultivated with *Agrobacterium* carrying either pAT4 – or pRS1.2, and roots of the pAT4 – line with *Agrobacterium* carrying pGA-FLP. *In planta* FLP activity was detected in the root explants as blue sectors after staining with XGlc (cf. [12]). Figure 2 shows the appearance of roots staining positive, and Table 1 summarizes a typical experiment. Clearly, in this transient assay FLP can promote site-specific recombination.

The number of blue sectors per cm root explant varied widely from experiment to experiment. In experiments (unlike that of Table 1) where in addition wild-type roots were co-cultivated with $pAT4^-$, very occasionally one or a few stained sectors appeared, invariably at a frequency per cm < 1% that of the pGA-FLP roots. These sectors, which were not investigated further, might have resulted from somatic homologous recombination [2] between the two *FRT* in the unintegrated T-DNA construct. Alternatively, they might have reflected an endogenous site-specific recombinase activity that can recognize FRT.

For stable transgenic plants, we tested activity in two approaches, by starting with a transgenic FLP expression construct line and retransforming it with a reporter *FRT* target construct, and alternatively, by crossing a transgenic FLP expression line with a transgenic reporter *FRT* line. In the retransformation approach, the reporter plasmid pRS1.2 (Fig. 1) was transformed into a FLP expression line by selection for Phl^r (phleomycin resistance [24]). After co-cultivation, selection and regeneration, leaves of primary transformants were stained for Gus activity. For each of three such transformants 15 to 20 leaves were scored, of which ca 20% showed blue sectors like the one in Fig. 3. These sectors evidently arose during somatic growth of leaf tissue. By contrast, no stained leaves were seen among primary transformants selected from co-cultivation of pRS1.2 with a wild-type line.

In the alternative approach, a homozygous line

Fig. 2. FLP activity in transient assays. Transgenic root explants were stained with XGlc after co-cultivation. Left, roots from a plant carrying pGA-FLP co-cultivated with *Agrobacterium* carrying pAT4 – . Right, roots from a plant carrying pAT4 – co-cultivated with *Agrobacterium* carrying pGA-FLP.

Table 1. FLP activity in transient assays in a representative experiment. Root explants were co-cultivated with *Agrobacterium* as described in the text. Data are expressed as number of individual Gus$^+$ sectors over total cm of root explant scored. NT, not tested.

T-DNA construct in *Agrobacterium*	Genomic construct in *Arabidopsis* root explants		
	pGA-FLP	pAT4–	none
pRS1.2	15/50	NT	0/80
pAT4–	50/50	NT	NT
pGA-FLP	NT	50/50	0/200

carrying the expression construct pGA-FLP was crossed with a homozygous line carrying the reporter construct pAT4 – (Fig. 1). Five crosses were successful, and leaves from F1 progeny were stained. Table 2 shows that despite the small number scored, leaves from three of the five crosses gave sectors staining blue like those in Fig. 3; one cross gave in addition a completely blue leaf, presumably resulting from an inversion event that took place before initiation of the leaf primordium.

Thus it is clear from both approaches that in transgenic plants as in transient assays, FLP can promote site-specific inversion. As in general the recombination product depends only on whether the two *FRT* target sites are in inverted repeat, in direct repeat, or on separate chromosomes, there is every reason to believe that FLP recombinase, like the mechanistically similar Cre recombinase [3, 7, 8, 21, 26, 28], is capable of promoting de-

Fig. 3. FLP activity in a transgenic plant. The leaf is from a regenerating plant stained with XGlc. The plant was originally transformed with pGA-FLP, and a homozygous progeny plant was then retransformed with pRS1.2 (see text).

Table 2. FLP activity in transgenic plants. Transgenic plants homozygous for the indicated constructs were crossed as described in the text. For each cross, two leaves were scored from each of two F1 progeny plants.

Male	Female	Cross	Number of F1 leaves scored		
			all blue	blue sectors	no staining
pGA-FLP	pAT4–	1	1	3	0
		2	0	0	4
		3	0	0	4
pAT4–	pGA-FLP	4	0	4	0
		5	0	4	0

letion and translocation as well. Although FLP is a yeast enzyme and higher plant chromatin is somewhat different from yeast chromatin [31], that difference is clearly no bar to *in planta* activity.

Like Cre recombinase but in contrast to Gin [17], FLP had no overall effect on plant growth, although specific deleterious rearrangements would not have been detected. Judging from the limited staining seen in transient assays (Fig. 2), retransformed transgenic plants (Fig. 3) and F1 cross progeny, activity does not appear to be especially high. Further work will be necessary to determine parameters, optimize growth conditions and substrate configurations, compare meiosis with somatic growth, etc. As in the case of Cre, to which this provides a complementary alternative, rearrangements promoted by FLP *in planta* should be useful for such manipulations as restructuring of the plant genome and marking of somatic cells for lineage analysis.

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FLP recombinase in transgenic plants: constitutive activity in stably transformed tobacco and generation of marked cell clones in *Arabidopsis*

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Summary

FLP site-specific recombinase was expressed in stably transformed tobacco and *Arabidopsis*. FLP-expressing tobacco lines were crossed with other transformed tobacco lines that contained a stably integrated FLP recognition target construct(s). The target construct consisted of two directly-oriented FLP recognition targets (FRTs), flanking a hygromycin resistance cassette located between a GUS coding region and an upstream 35S CaMV promoter. Excision of the hygromycin resistance cassette by FLP-mediated recombination between FRTs brings the GUS coding region under the transcriptional control of the CaMV 35S promoter. In the absence of FLP-mediated recombination, the GUS gene is transcriptionally silent. GUS activity was observed in the progeny of all crosses made between FLP recombinase-expressing and target-containing tobacco lines, but not in the selfs of parents. The predicted recombination product remaining after excision was confirmed by PCR and Southern analysis. In *Arabidopsis*, inducible expression of FLP recombinase was achieved from the soybean *Gmhsp* 17.6L heat-shock promoter. Heat-shock induction of FLP expression in plants containing the target construct led to activation of constitutive GUS expression in a subset of cells, whose progeny, therefore, were GUS-positive. A variety of clonal sectors were produced in plants derived from seed that was heat-shocked during germination. The ability to control the timing of GUS activation was demonstrated by heat-shock of unopened flower heads which produced large sectors. It was concluded that heat-shock-induced expression of FLP recombinase provides a readily controllable method for generating marked clonal sectors in *Arabidopsis*, the size and distribution of which reflects the timing of applied heat-shock.

Introduction

Site-specific recombinases are becoming increasingly important as *in vivo* tools for manipulating DNA in higher eukaryotic hosts. The range of heterologous hosts in which site-specific recombinases have been shown to function efficiently includes insects (Golic and Lindquist, 1989; Morris *et al.*, 1991), mammals (Lakso *et al.*, 1992; O'Gorman *et al.*, 1991; Orban *et al.*, 1992; Sauer and Henderson, 1988) and plants (Dale and Ow, 1990; Lloyd and Davis, 1994; Odell *et al.*, 1990). The ability of foreign site-specific recombinases to operate in heterologous hosts means that a variety of recombinase-mediated *in vivo* DNA manipulations in higher eukaryotes are now possible (reviewed by Kilby *et al.*, 1993), including the activation or removal of transgenes (Bayley *et al.*, 1992; Golic and Lindquist, 1989; Odell *et al.*, 1990; Orban *et al.*, 1992; Russell *et al.*, 1992; Zou *et al.*, 1994), the marking of cell lineages (Golic and Lindquist, 1989; Harrison and Perrimon, 1993; Struhl and Basler, 1993; Xu and Rubin, 1993), the targeted integration of DNA into the genome (Albert *et al.*, 1995; Baubonis and Sauer, 1993; Fukushige and Sauer, 1992; O'Gorman *et al.*, 1991; Sauer and Henderson, 1990), and the generation of chromosomal rearrangements (Medberry *et al.*, 1995; Osborne *et al.*, 1995; Qin *et al.*, 1994; Smith *et al.*, 1995).

Three related site-specific recombinases have been exploited in higher eukaryotes, Cre from bacteriophage P1 (Austin *et al.*, 1981), FLP from the 2 μm circle plasmid of budding yeast (*Saccharomyces cerevisiae*; Broach *et al.*, 1982), and R recombinase from the pSR1 plasmid of the yeast *Zygosaccharomyces rouxii* (Araki *et al.*, 1985). In their natural host environment these recombinases serve to resolve plasmid dimers into monomers (Cre) or to invert plasmid sequences (FLP and R). All, however, share properties of catalysing base-perfect recombination reactions between pairs of corresponding dsDNA target sites, without a requirement for co-factors or other proteins. The minimum target site recognized by, for example, FLP recombinase, is 34 bp (McLeod *et al.*, 1986) although shorter sequences down to 22 bp retain the ability to recombine (Jayaram, 1985; Proteau *et al.*, 1986; Senecoff *et al.*, 1985). In common with Cre and R recombinase target sites, FLP recognition target sites (FRTs) are asymmetrical (McLeod *et al.*, 1986; Senecoff and Cox, 1986) which gives them a sense of directionality. If FRT sites resident on linear dsDNA are arranged in indirect orientation relative to one other, interlying DNA between the FRT sites will be inverted (Figure 1a; Broach and Hicks, 1980). If however, FRT sites are arranged in direct orientation, interlying dsDNA is excised in circular form (Figure 1b; Vetter *et al.*, 1983).

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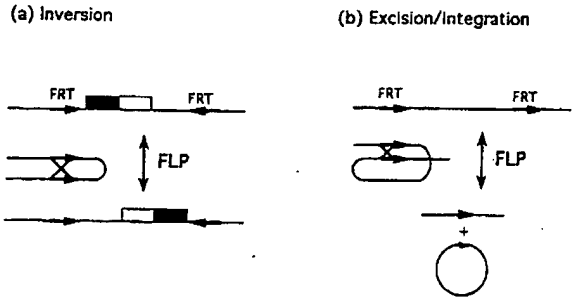

Figure 1. Possible outcomes of FLP-mediated site-specific recombination between two FLP recognition target (FRT) sites resident on linear dsDNA. (a) With FRT sites in head-to-head (indirect) orientation, interlying dsDNA is inverted. (b) With FRT sites in head-to-tail (direct) orientation, interlying dsDNA is excised (forward reaction) and resolved as a dsDNA circular molecule containing a single FRT site. As a consequence of the excision reaction, a single FRT site remains on the linear strand of dsDNA. The reverse of the excision reaction allows the targeted integration of double-stranded circular DNA into linear dsDNA at a site defined by location of a single FRT.

The characteristics of site-specific recombinases lend themselves to the introduction of these enzymes into heterologous organisms where experimentally introduced target sites appear to provide the only available substrate (reviewed by Kilby *et al.*, 1993). In transgenic plants, Cre recombinase has been used to activate gene expression (Bayley *et al.*, 1992; Odell *et al.*, 1990, 1994; Russell *et al.*, 1992), remove marker genes (Bayley *et al.*, 1992; Dale and Ow, 1991; Russell *et al.*, 1992), target gene integration (Albert *et al.*, 1995) and to generate chromosomal rearrangements (Medberry *et al.*, 1995; Osborne *et al.*, 1995; Qin *et al.*, 1994; reviewed by van Haaren and Ow, 1993). In contrast, R and FLP recombinases have been less widely exploited in plants. R recombinase has, however, been shown to function in transgenic tobacco (Onouchi *et al.*, 1991), and FLP activity has been demonstrated in transfected protoplasts of rice and maize (Lyznik *et al.*, 1993). More recently, FLP recombinase has been shown to function efficiently in the progeny of crosses made between primary transformed tobacco plants (Lloyd and Davis, 1994); these authors were, however, unsuccessful in their attempts to obtain transgenic *Arabidopsis* plants expressing FLP recombinase.

In *Drosophila*, FLP site-specific recombinase has proved particularly valuable for generating genetic mosaics and marking cell lineages (Dang and Perrimon, 1992; Golic, 1991; Golic and Lindquist, 1989; Harrison and Perrimon, 1993), and for studying the cell autonomy of gene products (Struhl and Basler, 1993). The general strategy adopted in these studies has been to place FLP recombinase under the control of a heat-shock responsive promoter and to use heat-shock-induced, transient FLP activity to activate constitutive expression of a marker gene. The marker gene remains active in all progeny cells derived from the progenitor in which FLP-mediated marker gene activation occurred. The usefulness of this method of marking cells derives principally from the use of a heat-shock promoter to control recombinase activity, thus allowing the timing of clonal marking to be experimentally determined.

Considerable benefits have been bestowed on lineage analysis in *Drosophila* by providing controlled expression of recombinase to activate a marker gene. The establishment of an equivalent system in plants would appear, therefore, to be a useful addition to those techniques currently available for lineage analysis. We note that at present, lineage studies in plants are, in the main, restricted to the use of periclinal chimeras (Marcotrigiano and Bernatzky, 1995; reviewed Tilney-Bassett, 1986), or to the use of X-irradiation to induce homozygous mutant sectors in plants heterozygous for a recessive marker gene. Although this latter approach has been used successfully to generate cell fate maps in, for example, *Arabidopsis* (Furner and Pumfrey, 1992, 1993), X-irradiation induces a relatively low frequency of chimerism and can cause cell damage. Other approaches that have been used to generate marked sectors in plants include micro-injection of a marker gene into individual cells (Lusardi *et al.* 1994) and the induction of rare intrachromosomal recombination events (Swoboda *et al.*, 1994).

In work described here we extend the initial observations of Lloyd and Davis (1994) on the constitutive activity of FLP recombinase in transformed tobacco, and also show that FLP recombinase can be expressed in *Arabidopsis*. We present a controllable and readily manipulated method for generating marked sectors in *Arabidopsis* using FLP site-specific recombinase to activate β-glucuronidase (GUS) gene expression in appropriately transformed plants. Control of transient recombinase expression is achieved by using the soybean *Gmhsp* 17.6L heat-shock promoter (Severin and Schöffl, 1990), which allows the timing of sectoral marking to be experimentally determined. We also propose the potential of this system for the analysis of gene function by activation or removal of gene function within clonal sectors of chimeric plants.

Results

Strategy for FLP-mediated activation of GUS expression

The excision reaction of FLP recombinase (Figure 1b, forward direction) can be used to remove a sequence which has been placed between a promoter and a downstream gene; the interlying sequence having originally being incorporated to prevent gene transcription. Removal of this blocking sequence or 'excision cassette' by FLP thus allows expression of a downstream marker (Lloyd and Davis, 1994). The blocking sequence can contain a second marker, so that recombination results in a 'marker swap'. This type of arrangment has been used in plants with Cre

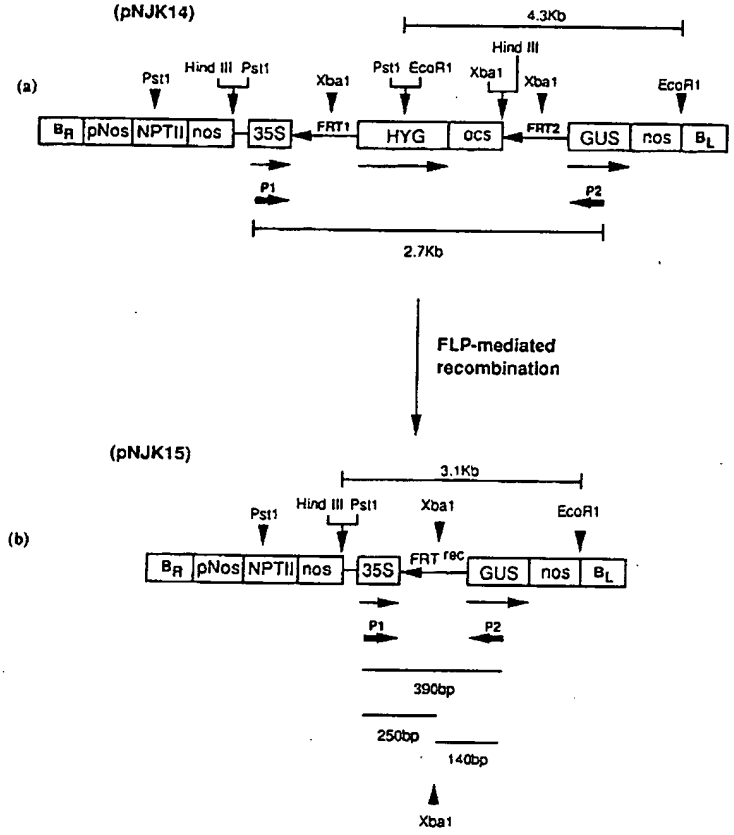

Figure 2. FLP recombinase target construct prior to/post FLP-mediated recombination between FLP recognition target sites.
(a) FLP recombinase target construct (pNJK14) prior to FLP-mediated excision of interlying dsDNA resident between FLP recognition target (FRT) sites. (b) Excision product (pNJK15) remaining after FLP-mediated recombination between FRT sites resident on pNJK14. Recombination between FRT sites resident on pNJK14 (i.e. FRT1 and FRT2) results in the formation of a recombined FRT site (comprising half of FRT1 and half of FRT2, but identical to the parent sites) designated here as FRT^{rec}. Transgenic plants containing pNJK14 are resistant to hygromycin, but are GUS-negative because the GUS coding region (GUS) lacks a promoter. FLP-mediated excision of the hygromycin coding region (HYG) brings the 35S CaMV promoter originally used to drive the hygromycin coding region immediately 5′ to the GUS coding region, causing GUS expression in plants in which an excision event has occurred. Predicted PCR product sizes corresponding to forward (P1) and reverse (P2) primers, are shown. The predicted sizes of the two fragments produced after digestion of the 390 bp PCR product with *Xba*I are indicated. B_L, left T-DNA border; B_R, right T-DNA border; pNos, nopaline synthase promoter; 35S, 35S cauliflower mosaic virus promoter; HYG, hygromycin coding region; GUS, β-glucuronidase coding region; NPT-II, neomycin phosphotransferase II coding region; ocs, 3′ transcription termination sequences of the octopine synthase gene; nos, 3′ transcription termination sequences of the nopaline synthase gene.

(Bayley *et al.*, 1992; Odell *et al.*, 1994) and FLP recombinase (Lloyd and Davis, 1994), although only in the work of Odell *et al.* (1994) was the downstream gene a cell autonomous marker suitable for analysis of chimeric plants.

We have adopted a strategy for FLP-mediated marker gene activation in plants similar to that reported for marker gene activation in *Drosophila* (Struhl and Basler, 1993). Our strategy involves transformation of plants with a construct in which the GUS coding region is separated from a constitutive promoter by a cassette containing a marker gene and a polyadenylation sequence that blocks transcription of the GUS gene. The cassette is flanked by FRTs arranged in direct orientation. Expression of functional FLP recombinase protein is predicted to cause excision of the blocking sequence by site-specific recombination between the FRTs, bringing the GUS coding region immediately adjacent to its previously distal promoter (Figure 2).

We chose to introduce FLP recombinase and target molecules into the same genetic background by cross-fertilization of plants containing the target construct with plants competent for FLP expression. This method of bringing a recombinase source and recombinase target together in plants is an established procedure (Bayley *et al.*, 1992; Odell *et al.*, 1990, 1994; Lloyd and Davis, 1994; Russell *et al.*, 1992).

Production of transgenic tobacco lines expressing FLP recombinase and lines containing target constructs

Tobacco plants were transformed with a construct expressing FLP recombinase (termed Δ FLP) under the control of a constitutive cauliflower mosaic virus (CaMV) 35S promoter (Figure 3a; see Experimental procedures). Further lines were produced containing the target construct pNJK14 (Figure 2a), which carries the GUS gene separated from a CaMV 35S promoter by an excision cassette, consisting of the hygromycin phosphotransferase coding region and 3′ transcription termination sequences from the octopine synthase gene, flanked by directly oriented FRT sites.

Additional transformed plants were produced containing a positive control construct pNJK15, equivalent to the target construct after a successful FLP-mediated excision reaction (Figure 2b). This was to confirm that GUS transcription *in planta* could be driven from the 35S CaMV promoter through the FRT that remains after excision (see Figure 7a).

(a)

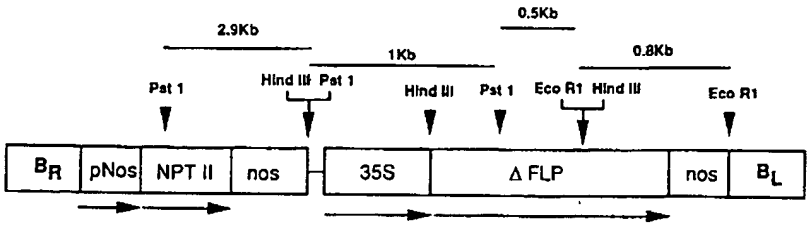

Figure 3. Structural (T-DNA) arrangement of constitutive and inducible sources of FLP recombinase.
(a) Constitutive source of FLP recombinase constructed using a modified FLP recombinase coding region (see text; designated here as Δ FLP) driven by a 35S CaMV promoter.
(b) Inducible (heat-shock) source of FLP recombinase constructed using the native FLP coding region driven by the soybean *Gmhsp* 17.6L heat-shock (HSP) promoter.
Restriction sites and corresponding predicted restriction fragment lengths were used to assess minimum insert number and construct integrity *in planta* by Southern analysis. Abbreviations are the same as in the legend to Fig.2.

(b)

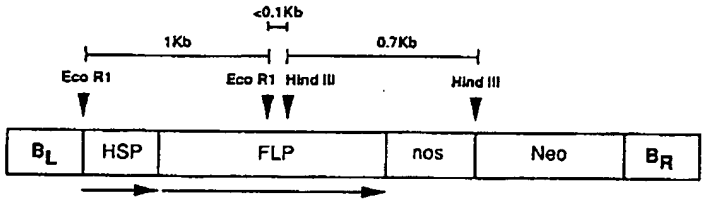

Table 1. Segregation of kanamycin resistance in 35S Δ FLP and FLP target-containing tobacco plants as determined using the vertical plate root length assay

		Observed segregation			Predicted no. if segregating (3:1)		Predicted no. if segregating (15:1)		
Generation	Plant line	LR	SR	Total	LR	SR	LR	SR	Chi squared
T_1	Δ FLP No. 1	18	7	25	18.75	6.25	–	–	0.12 ^{ns}
T_1	Δ FLP No. 2	16	8	24	18	6	–	–	0.88 ^{ns}
T_1	Target No. 1	41	2	42	–	–	–	–	ND
T_1	Target No. 3	29	9	38	28.5	9.5	–	–	0.034 ^{ns}
T_1	Target No. 6	20	9	29	21.75	7.25	–	–	0.562 ^{ns}
T_2	Δ FLP No. 1	18	7	25	18.75	6.25	–	–	0.12 ^{ns}
T_2	Δ FLP No. 2	16	8	24	18	6	–	–	0.888 ^{ns}
T_2	Target No. 1	84	6	90	–	–	84.37	5.62	0.026 ^{ns}
T_2	Target No. 3	43	12	55	41.25	13.75	–	–	0.293 ^{ns}
T_2	Target No. 6	39	12	51	38.25	12.75	–	–	0.058 ^{ns}

T_1 tobacco seedlings were from selfs of primary transformed plants and T_2 seedlings were from selfs of T_1 plants. Seedlings were scored as resistant (long root; LR) or sensitive (short root; SR) after 2 weeks growth on DMS medium containing 100 mg l^{-1} kanamycin sulphate. ^{ns}, not significant at the 5% level; ND, not determined.

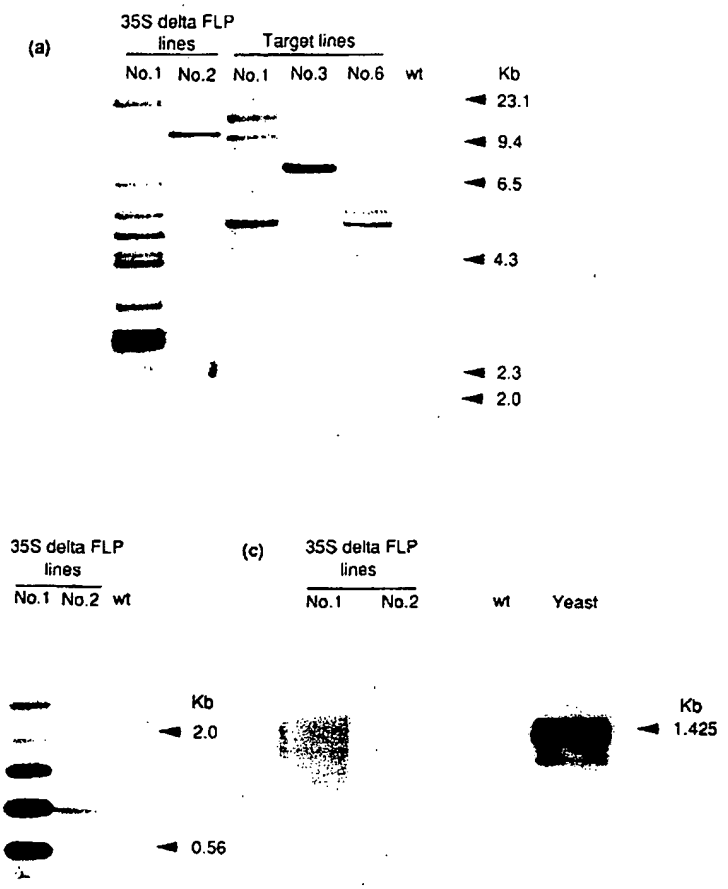

Figure 4. Southern and Northern analysis of transformed tobacco lines used in constitutive FLP expression studies.
(a) Minimum insert number of 35S Δ FLP source- and target (pNJK14)-containing lines. DNA samples from pooled T_1 generation plants were digested with *Hin*dIII. The blot was probed with the entire NPT II coding region isolated as a 1.1 kb *Hin*cII/*Kpn*I fragment from pJC5 (Lichtenstein, personal communication).
(b) Integrity analysis of 35S Δ FLP source lines. DNA samples were digested with *Pst*I and *Eco*RI. The blot was probed with the entire Δ FLP coding region isolated as a 1.3 kb *Bam*HI/*Sst*I fragment from pEMBL131$^+$ Δ FLP (this paper).
(c) Comparison of mRNA transcripts isolated from 35S Δ FLP source lines with native mRNA transcripts from yeast; mRNA isolated from wild-type (wt) plants was used as a control. The probe used was as described in 4b, above.

In all cases, primary transformed (T_0 generation) plants were allowed to self (T_1 generation), and segregation ratios for linked selectable marker genes were determined for the progeny of selfed T_0 and T_1 plants by root length assay (Table 1). All putative transformants gave segregation ratios by chi-squared analysis consistent with one or two active selectable marker inserts per transformant; segregation patterns were stably inherited.

Southern analysis of DNA from pooled T_1 plants (Figure 4a and b) revealed that in some transformed lines the insert distribution was more complex than the phenotypic data suggested. For example, segregation analysis of 35S Δ FLP line No. 1 (see Table 1) indicated that there was only one active T-DNA, whereas Southern analysis (Figure 4a) suggested a minimum of 10 inserts were present in the genome. These extra inserts not revealed by segregation analysis presumably represent either additional tightly linked functional inserts, and/or linked or unlinked silent, or partially deleted inserts. Nevertheless, Northern analysis of total RNA from FLP transformed lines (Figure 4c) showed that a FLP transcript of comparable size to the native yeast FLP message was present in all Δ FLP source plants.

T-DNA integrity analysis of Δ FLP source lines (Figure 4b) revealed the presence of all the predicted restriction fragments (1.0, 0.8 and 0.5 kb) for the *Pst*I/*Eco*RI digest and FLP probe used, providing evidence that at least one 35S Δ FLP–nos element in each line was structurally intact. The additional bands observed in the integrity blot that correspond to 35S Δ FLP source line No. 1 (Figure 4b, lane 1) presumably represent rearranged T-DNA copies also present in this multiple insert line. The stoichiometry of the bands observed in the integrity blot for 35S Δ FLP source line No. 2 (Figure 4b, lane 2) reflects the length of homologous sequence shared between the probe used and each of the three 35S Δ FLP-nos element fragments produced by restriction with *Pst*I and *Eco*RI. It is possible that the restriction fragments observed, although corresponding to the predicted sizes of bands from intact inserts, were obtained from different inserts, each of which had part of the T-DNA intact. However, at least in the case of 35S Δ FLP source line No.2, where no additional restriction fragments were revealed by Southern analysis (Figure 4b, lane 2), we consider this unlikely. This conclusion is supported by the GUS activity we observed in the progeny of crosses made between all 35S Δ FLP source lines and target-containing plants (see below), providing strong evidence of

Table 2. Segregation of the GUS phenotype in the progeny of crosses made between T_1 generation Δ FLP source and FLP target (pNJK14) containing tobacco lines; all progeny from the selfs of parents were GUS-negative

	Observed segregation			Predicted No. if segregating 3:1		
Cross	GUS^+	GUS^-	Total	GUS^+	GUS^-	Chi squared
Target no. 1 × Δ FLP no. 1	35	8	43	32.25	10.75	0.937 ^{ns}
Δ FLP no. 1 × Target no. 1	32	9	41	30.75	10.25	0.202 ^{ns}
Target no. 1 × Δ FLP no. 2	20	7	27	20.25	6.75	0.012 ^{ns}
Target no. 3 × Δ FLP no. 1	115	0	115	–	–	ND
Target no. 6 × Δ FLP no. 1	33	18	51	38.25	12.75	2.881 ^{ns}

An observed 3:1 (GUS^+:GUS^-) segregation ratio in the progeny of a cross is consistent with the view that one of the parents was homozygous for one active FLP gene or GUS gene, and the other parent was hemizygous for two active FLP genes or GUS genes. If both parents in a cross are homozygous, all progeny from that cross are predicted to be GUS^+. ^{ns}, not significant at the 5% level; ND, not determined; (GUS^+), GUS-positive; (GUS^-), GUS-negative.

FLP activity and hence the structural integrity of the requisite portions of the FLP source and target T-DNAs.

Analysis of target lines showed that only one line (target line No. 3) unequivocally contained a single active selectable marker insert (Table 1 and Figure 4a). Analysis was continued with lines containing both multiple and single inserts, since, although the genetic analysis is simplified by use of single insert-containing lines, the presence of multiple targets increases the probability of observing a successful excision event.

In order to demonstrate that GUS expression would be observed from the excised construct, germinated seedlings from the T_1 generation of a representative positive control plant were stained with X-Gluc and examined for GUS activity (see Experimental procedures). All positive control tobacco seedlings examined (> 100) were strongly GUS-positive, exhibiting an even distribution of stain throughout all vegetative structures with the exception of the root tip (see Figure 7a). This confirmed that the recombinant target product produced after FLP-mediated excision would be competent for GUS expression. Similar staining was also carried out of seedlings from lines carrying the pNJK14 target excision construct. All such seedlings (>150) were GUS-negative (data not shown), indicating that there was no detectable transcriptional readthrough into the GUS gene prior to the predicted excision event. Moreover, in all subsequent experiments carried out in tobacco (and in *Arabidopsis*) we have never observed a GUS-positive reaction in target-containing plants unless an active source of FLP recombinase was also present.

Activity of FLP recombinase in transgenic tobacco

Plants from all T_1 generation target lines transformed with pNJK14 were crossed to T_1 generation 35S Δ FLP source plants, using the target lines as female parents; one reciprocal cross was also made between target line No.1 and 35S Δ FLP source No. 1. Vernalized seeds from crosses, and from the selfs of parents, were germinated on DMS medium, allowed to grow for 2 weeks, then stained with X-Gluc. GUS-positive seedlings were observed in the progeny of all the crosses made, indicating successful FLP-mediated target excision (Table 2 and see Figure 7b), but were not observed in any of the progeny of selfed parents (data not shown). This observation confirmed that the target construct behaved as predicted in the presence of

FLP recombinase, and demonstrated the efficient operation of FLP recombinase *in planta*.

The cross between target line No. 6 and 35S Δ FLP source line No. 1 was chosen for more detailed molecular analysis using PCR and Southern blot analysis. Individual, randomly selected, *in vitro* grown progeny seedlings, and selfs of parents, were tested for their GUS phenotypes (staining of a sacrificed portion of true leaf with X-Gluc) then transferred from tissue culture to soil. Total genomic DNA was isolated from seven representative GUS-positive and three GUS-negative plants, and from both a bulked unexcised target line population and a bulked positive control line containing pNJK15. Each DNA sample was then used for PCR analysis (Figure 5a), using primers specific to the 3′ end of the 35S CaMV promoter (forward primer) and to the 5′ end of the GUS coding region (reverse primer). The unexcised (control) target line gave a single PCR product of predicted size 2.7 kb (see Figure 2a). The positive control line containing the equivalent of the excised construct gave a single PCR product of predicted size 0.39 kb (see Figure 2b). PCR amplification of DNA from all seven GUS-positive lines gave a single PCR product identical in size to that expected for a correct FLP-mediated excision reaction (0.39 kb); no PCR product was detected in DNA isolated from GUS-negative lines or from wild-type DNA. These data are consistent with the view that the GUS-positive phenotype segregating in the progeny from the cross was the result of an excision reaction or rearrangement that brought the distal 35S CaMV promoter adjacent to the GUS coding region in a transcriptionally correct orientation. To confirm that this was actually as a result of base-perfect recombination between FRT sites, the PCR excision product from GUS-positive lines was subjected to restriction analysis (Figure 5b). Correct FLP-mediated excision recreates an *Xba*I site located at the cross-over site in the core of the FRT (McLeod *et al.*, 1986), producing predicted *Xba*I restriction fragment sizes for the PCR excision product described here, of 250 and 140 bp (see Figure 2a and b), as shown in Figure 5(b).

Further confirmation of FLP-mediated excision was obtained by Southern blot analysis (Figure 5c) of DNA from two of the selected GUS-positive and one of the selected GUS-negative progeny plants as compared with DNA from the (un-excised) parental target line. For the

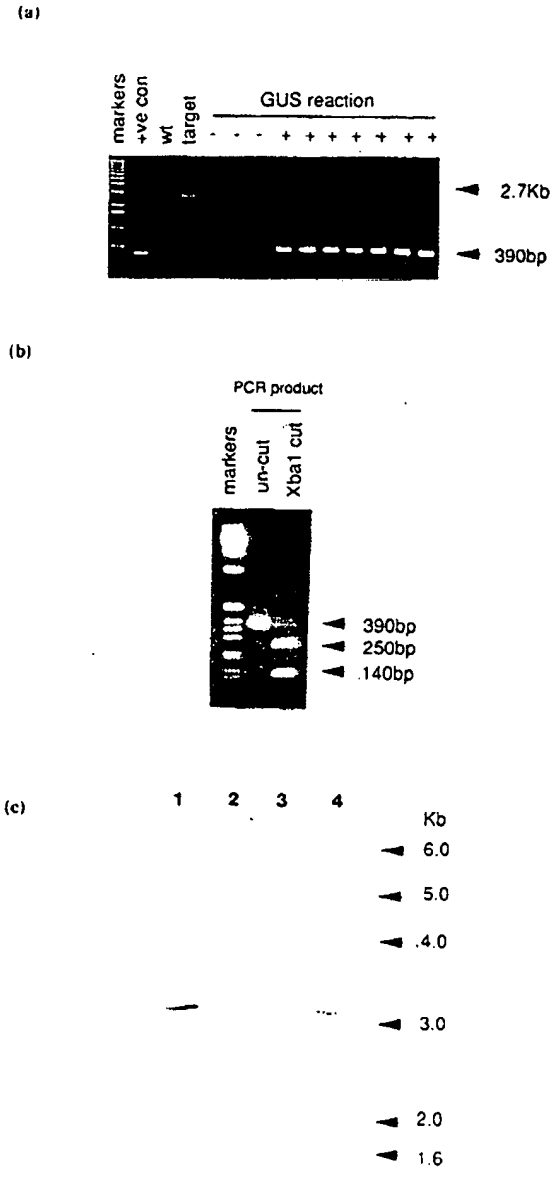

Figure 5. PCR and Southern blot analysis of target excision events in tobacco plants.

(a) DNA isolated from individual, GUS phenotyped progeny collected from a cross (as shown in Figure 7b) between tobacco plants containing the target construct pNJK14 (target line No. 6) and plants containing a constitutive source of FLP recombinase (35S Δ FLP line No. 1) was subjected to PCR analysis. Forward and reverse primers were designed to amplify either a 390 bp PCR product indicative of FLP-mediated target excision and/or a 2.7 kb PCR product indicative of unexcised target. DNA from a bulked population of transformed plants containing the excision-modified positive control construct (pNJK15), and a bulked population of target line selfs and wild-type (wt) plants were used as controls. (+), GUS-positive. (–), GUS-negative. The absence of a PCR reaction product in GUS-negative plants is consistent with the target-containing parent segregating for the construct.

(b) Restriction analysis of isolated PCR excision product. Base-perfect recombination between FRT sites resident on the target construct pNJK14 is predicted (see Figure 2b) to yield a 390 bp PCR product with an asymetrically located *Xba*I restriction site. The predicted size of the two fragments produced after digestion of this PCR product with *Xba*I, are 250 and 140 bp.

(c) Southern analysis of target excision; lanes 1–4. DNA from two GUS-positive plants (lanes 1 and 4), one GUS-negative plant (lane 3) and from the parental target line (lane 2) was restricted with *Eco*RI and *Pst*I then probed with the entire GUS coding region isolated as a 1.8 kb *Sma*I/*Sst*I fragment from pC–TAK1 (Kavanagh, personal communication). Hybridization of the probe to a 4.3 kb restriction fragment is indicative (see Figure 2a) of unexcised target. Hybridisation of the probe to a 3.1 kb restriction fragment is indicative (see Figure 2b) of target excision. Where both 3.1 and 4.3 kb restriction fragments are detected, this indicates that target excision occurred, but that not all resident target molecules were excised.

GUS probe and *Eco*RI/*Pst*I digest used, the prediction is (see Figure 2a and b) that in the absence of FLP-mediated target excision a single 4.3 kb restriction fragment would be detected. This was observed only in the case of DNA corresponding to the parental target line (Figure 5c, lane 2). In the event of successful FLP-mediated excision of all target inserts, the prediction is (see Figure 2a and b) that a 3.1 kb restriction fragment would be detected. DNA from GUS-positive progeny plants yielded both 4.3 kb and 3.1 kb restriction fragments (Figure 5c, lanes 1 and 4). These data imply that in the progeny of the cross, FLP-mediated target excision occurred, but that not all target inserts were excised. The presence of unexcised target inserts may reflect the inability of FLP recombinase to excise certain targets, or a lower level of expression of FLP recombinase in some cells. The absence of any hybridization of the probe to DNA from the GUS-negative progeny plant (Figure 5c, lane 3) suggests that, in this line at least, the target was not inherited. No PCR product equivalent to the unexcised target was seen in any of the GUS-negative lines examined by PCR (Figure 5a, lanes 5–7). Given the combination of the Southern blot data and the fact that it was possible to amplify the 2.7 kb PCR reaction product predicted for an unexcised target molecule (Figure 5a, lane 4), we conclude that in the GUS-negative progeny lines from the cross, the target(s) had not been inherited, presumably as a result of segregation at meiosis.

To confirm that the FLP-recombined GUS phenotype could be stably inherited, seedlings from the selfs of two FLP-recombined GUS-positive plants (from the cross between target line No. 6 and 35S Δ FLP source line No.1) were stained for GUS activity. Forty out of 43 seedlings from one such plant and 47 out of 50 seedlings from the other plant were GUS-positive. These segregation data are consistent with the view (see legend to Table 2) that in the original cross between target line No. 6 and 35S Δ FLP source line No. 1, the target line parent, although possessing only one active selectable marker gene (Table 1) was hemizygous for two active GUS genes.

Heat-shock induced expression of FLP recombinase in Arabidopsis

To generate *Arabidopsis* lines competent for transient expression of FLP recombinase, plants were transformed with the native FLP recombinase coding region driven by the soybean *Gmhsp* 17.6L heat-shock promoter (HSP FLP). Transformed plants were screened using Northern analysis for the ability to produce a FLP recombinase transcript in response to heat-shock (4 h, 37°C). One line was selected (designated AraHSF1) for use in clonal sector generation experiments as a heat-shock-induced transient source of FLP recombinase. Segregation analysis indicated that AraHSF1 contained one active insert ($\chi^2 = 1.384$, $0.2 < P < 0.25$) with a minimum insert number, as deduced by Southern analysis, of 3 (Figure 6a). T-DNA integrity analysis showed that AraHSF1 contained at least one intact copy of the HSP- FLP-nos expression construct (Figure 6b, see also Figure 3b for prediction of restriction fragment sizes). Northern analysis (Figure 6c) showed that after heat-shock (37°C, 4 h) of AraHSF1 a transcript comparable in size to native yeast FLP message was induced.

To generate target-containing *Arabidopsis* lines, plants were transformed with the target construct pNJK14. One of the transformed lines (line B) segregated two active inserts ($\chi^2 = 0.710$, $0.3 < P < 0.5$) with a minimum insert number, as deduced by Southern analysis, of more than or equal to 5 (Figure 6a). Line B was used as the target line in all clonal sector studies.

To confirm that the target construct pNJK14 would direct GUS expression in all vegetative structures of *Arabidopsis* after FLP-mediated recombination/excision, *Arabidopsis* plants were transformed with the positive control construct pNJK15. Staining seedlings from such lines with X-Gluc showed that GUS was expressed in all vegetative structures, including the full length of the root (Figure 7c).

Generation of clonal sectors

To produce experimental material for clonal sector analysis, the heat-shock FLP source line AraHSF1 was crossed to FLP target line B. Preliminary studies concentrated on demonstrating the generation of clonal sectors by use of repeated heat-shock (4 h at 37°C per treatment). After vernalization (5 days at 4°C), *in vitro* grown progeny seedlings from crosses were heat-shocked every day for 1 week, then allowed to grow for another week prior to staining with X-Gluc. This procedure produced approximately 1 in 10 plants with clonal GUS sectors that were of a variety of shapes, sizes and distribution (see Figure 7d–f); no sectors were observed in plants not subjected to heat-shock. The sectoring produced was consistent with the prediction that multiple rounds of heat-shocked-induced, FLP-mediated GUS switching would result in both early and late excision events in treated plants, small sectors being derived from late recombination events or from recombination events in cells that subsequently only underwent a few cell divisions, and large sectors being derived from early recombination events. PCR analysis of DNA extracted from both heat-shocked and un-heat-shocked sibling plants (Figure 6d) showed that only in the case of heat-shock treatment was a PCR excision product of 0.39 kb detected. GUS-positive sectors thus arose as a consequence of FLP induction.

In order to generate clonal sectors initiated early in development, whole plants (hemizygous for both AraHSF1 and target line B) with floral structures at all stages of development were heat-shocked (37°C, 4 h). Seed was

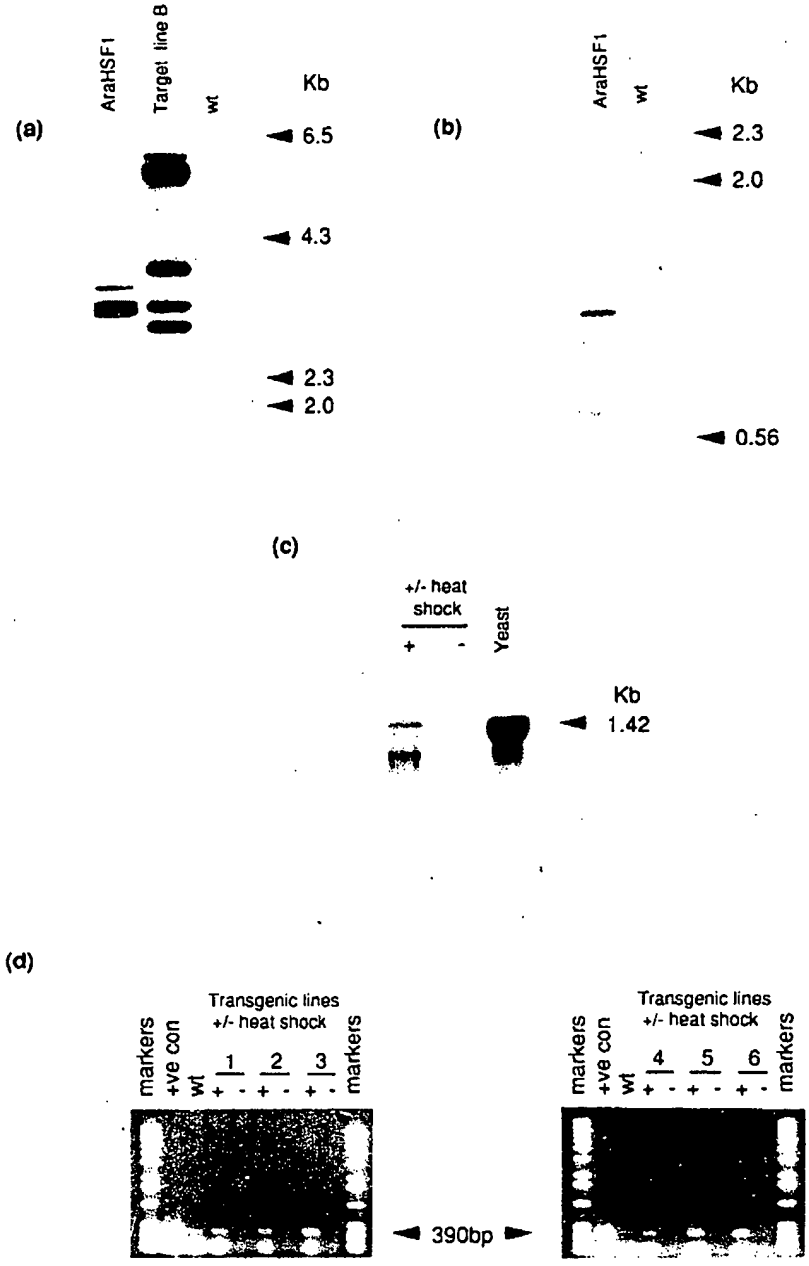

Figure 6. Southern and Northern analysis of transformed *Arabidopsis* lines used in clonal sector experiments, and PCR target excision analysis of heat-shocked plants that gave clonal sectors.

(a) Minimum insert number of heat-shock (HSP) FLP source line (designated AraHSF1) and target-containing line (line B). DNA from the HSP FLP source line was digested with *Eco*RI. DNA from the target line was digested with *Hin*dIII. Blots were probed with the entire NPT-II coding region isolated as described in the legend to Figure 4a.

(b) Integrity analysis of the HSP FLP source line. DNA was digested with *Hin*dIII and *Eco*RI. The blot was probed with the entire native FLP coding region isolated as a 1.3 kb Asp718/*Sst* fragment from pEMBL131$^+$ FLP (pMS11; this paper).

(c) Transcript analysis of FLP message produced by AraHSF1 in response to heat-shock (37°C, 4 h) compared with native yeast FLP message; mRNA from wild-type (wt) plants was used as a control.

(d) PCR excision assay performed on DNA isolated from six independent transgenic *Arabidopsis* lines containing an inducible (heat-shock) source of FLP recombinase and target construct. Plants produced clonal GUS sectors in reponse to heat-shock (+), sectors being visualized after a 1 week period of growth post-heat-shock treatment (see text); no GUS sectors were observed in un-heat-shocked (–) siblings. Only in the case of heat-shock was a 390 bp PCR product, indicative of target excision, detected. PCR conditions and primers are as described in the legend to Figure 5. wt, wild-type DNA; +ve con, DNA from a bulked population of positive control plants transformed with the excised target construct, pNJK15.

collected from individual siliques, vernalized (4°C, 5 days), germinated on DMS medium, and the seedlings so obtained were stained with X-Gluc after 2 weeks of growth. The various stages of flower/ silique development represented at the time of heat-shock fell broadly into three categories; (i) immature, ripening siliques; (ii) open flower heads with mature pollen; and (iii) unopened flower heads. Large clonal GUS sectors were observed in seedlings grown from seed originating from flower heads that were unopened at the time of heat-shock (Figure 7g and h); no clonal GUS sectors were observed in any seedlings grown from seed taken from heat-shocked, immature ripening siliques or from heat-shocked, open flower heads.

Discussion

FLP recombinase can catalyse recombination between experimentally introduced FRT sites in cells of a wide range of heterologous species, including *Escherichia coli* (Cox, 1983; Preibisch *et al.*, 1984), *Drosophila* (Golic and Lindquist, 1989), mammalian cells (O'Gorman *et al.*, 1991), mosquito (Morris *et al.*, 1991), protoplasts of maize and

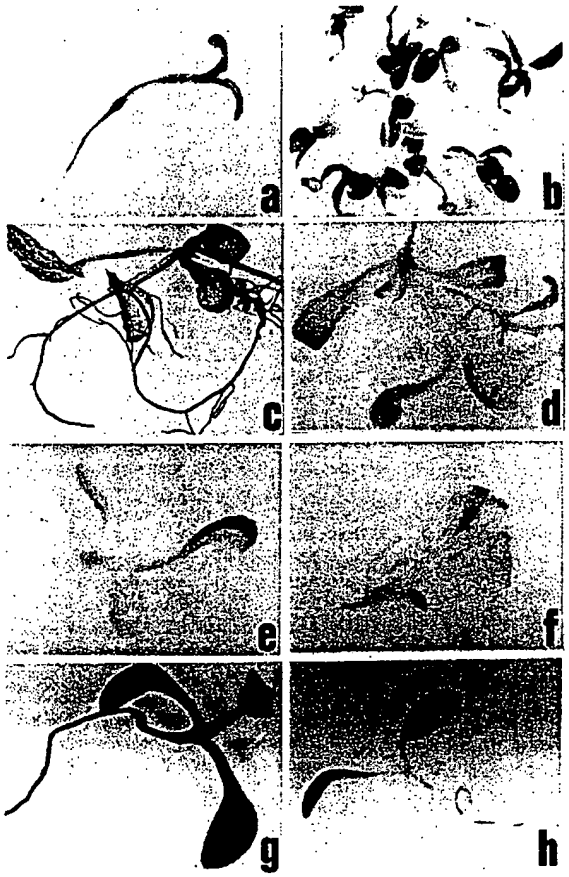

Figure 7. GUS activity in transformed tobacco and *Arabidopsis* seedlings as revealed by histochemical staining with X-Gluc.
(a) Constitutive expression of GUS in a tobacco seedling transformed with the positive control construct, pNJK15; note the absence of staining in the root tip.
(b) Segregation of the GUS phenotype in the progeny of a cross between a tobacco line expressing a constitutive source of FLP recombinase and a line containing the FLP target construct, pNJK14.
(c) Constitutive expression of GUS in an *Arabidopsis* seedling transformed with the positive control construct; note that all vegetative structures exhibit GUS activity.
(d–f) Heat-shock-induced clonal GUS sectors in *Arabidopsis* seedlings that were grown and heat-shocked *in vitro*. Progeny from a cross between an *Arabidopsis* line (AraHSF1) competent for inducible (heat-shock) expression of FLP recombinase and a FLP target-containing line were heat-shocked (37°C, 4 h) on a repeated basis (every day for a week after vernalization), then stained for GUS activity 1 week later. Small clonal GUS sectors are indicative of a somatic FLP-mediated excision event that activated GUS expression in founder cells which only undertake a limited number of subsequent divisions, whilst large clonal GUS sectors are indicative of GUS activation events that occurred early in development in cells giving rise to extensive lineages.
(g and h) Clonal GUS sectors in 2-week-old seedlings derived from seed collected from a heat-shocked greenhouse-grown plant that contained an inducible (heat-shock) source of FLP recombinase and the FLP target construct. Heat-shocking of the whole plant was at 37°C for 4 h. Siliques from which seed was collected originated from flower heads that were unopened at the time of heat-shock.

rice (Lyznick *et al.*, 1993) and tobacco plants (Lloyd and Davis, 1994). Here we extend the range of species in which FLP recombinase has been shown to catalyse site-specific recombination between locally arranged FRTs to include *Arabidopsis*. We also present an analysis of FLP recombinase activity in tobacco, which confirms and elaborates the work of Lloyd and Davis (1994). We note that tobacco plants that express FLP recombinase offer an eminently suitable background in which to test the operation of FLP recognition-based target constructs prior to their transformation and use in other plant species whose competence to express FLP recombinase is untested.

Studies in *Drosophila* have shown that FLP recombinase can be used to generate marked sectors of cells by deletion of sequences located between directly oriented FRT sites (Golic and Linquist, 1989; Struhl and Basler, 1993) or by recombination between replicated non-sister chromatids of homologous chromosomes (Golic, 1991; Harrison and Perimon, 1993; Xu and Rubin, 1993). We have shown that the former strategy can be used to generate clonal GUS-positive sectors within otherwise GUS-negative *Arabidopsis* plants by using transiently expressed FLP recombinase to activate GUS expression in cells previously held silent for GUS activity. In addition, we have shown that the developmental time point at which GUS expression is initiated can be controlled by the timing of the heat-shock used to induce transient expression of FLP recombinase from a heat-shock responsive promoter. Transient FLP expression allows the controlled switching of GUS from an inactive to a constitutively active state by FLP-mediated removal of a transcription blocking sequence (flanked by directly oriented FRTs), that previously served to separate the GUS coding region from its promoter. This is analagous to strategies developed in *Drosophila* where hsp70-promoter-controlled FLP expression has been used to activate the cell autonomous marker, β-galactosidase (Struhl and Basler, 1993). Importantly, data presented here show that the recombined construct remaining after FLP-mediated excision of our target construct was competent for GUS expression in *Arabidopsis*, and that GUS expression could occur from this construct in all vegetative structures (Figure 7c). This demonstrates that GUS activation is potentially suitable for following cell lineages in any plant structure in *Arabidopsis*, particularly since strict cell autonomy of GUS staining is achievable by manipulation of the reaction conditions (Eady *et al.*, 1994; Stomp, 1992).

Initial clonal sector generation experiments described here used repeated heat-shock to induce clonal sectors in *Arabidopsis* seedlings grown *in vitro*. This procedure resulted in the generation of clonal sectors in approximately one out of every 10 heat-shocked seedlings; no sectors were observed in un-heat-shocked sibling plants. Some heat-shocked seedlings that did not produce sectors in response to heat-shock may not have inherited the

correct combination of permissive inserts. To avoid this in future experiments, we are currently segregating the inserts in parental lines, with the intention of producing single insert, homozygous bulked populations. Some of the GUS-negative plants we observed must, however, have inherited both heat-shock FLP and target constructs, indicating that FLP activity is not always sufficient to catalyse the excision reaction. We are, therefore, also screening for heat-shock FLP lines with elevated heat-shock-induced FLP expression levels.

Both large and small GUS sectors were observed in heat-shocked plants. The sector sizes in these experiments are indicative of the number of subsequent divisions of a cell in which GUS was activated. We propose that this is correlated with the time at which GUS activity is initiated, with small sectors (Figure 7d) arising either from cells in which GUS activity was activated by a later heat-shock or from cells which subsequently only underwent a limited number of additional rounds of cell division; extension of this argument implies that large sectors (Figure 7f) were derived from cells in which GUS activity was initiated early in development. Our view that sector size is idicative of the number of subsequent rounds of cell division that a cell in which GUS expression has been activated, then undergoes, is further supported by the observation that some repeatedly heat-shocked seedlings possessed both small and large sectors (Figure 7e). This result is entirely consistent with the conditions of heat-shock treatment used, i.e. multiple rounds of heat-shock given from seed germination to the formation of the first set of true leaves.

The most extensive clonal GUS sectors generated in any experiment were those produced as a result of heat-shock treatment of whole plants. Seed from siliques derived from flower heads un-opened at the time of heat-shock gave rise to seedlings with extensive GUS sector coverage (Figure 7g and h). As already mentioned, we suggest that this type of sectoring is indicative of GUS activation early in development. The lack of GUS sectoring in seedlings derived from seed collected from ripening siliques or opened flower heads with mature pollen, which were also present at the time of heat-shock, might be a result of the failure of the heat-shock promoter to induce sufficient FLP recombinase in these cells, or the inability of FLP recombinase to function in these cell types.

The results cited above confirm that heat-shock induction of FLP site-specific recombinase can be readily used to generate marked sectors of cells in *Arabidopsis*, with the timing of marker gene activation determined by the timing of an applied heat-shock. The *Gmhsp* 17.6L promoter used by us appears to have a widespread developmental expression in *Arabidopsis*. Indeed, the observation that extensive GUS sectoring events could be induced in both root and leaf tissues (Figure 7g and h) by application of heat-shock early in development (as detailed above) suggests that the *Gmhsp* 17.6L promoter can function in both root and apical meristems, thus allowing the possibility of generating sectoring events in any root/apical meristem-derived structure. Our data show, however, that the *Gmhsp* 17.6L promoter may not provide sufficient expression to catalyse FRT/FRT recombination in the developmental stages represented in open flowers or ripening siliques. Importantly, no GUS activation events were observed in the absence of heat-shock, and no constitutive GUS activity was seen in any *Arabidopsis* (or tobacco) transformant carrying only the target construct.

We note that the only previous report describing expression of a site-specific recombinase that resulted in sectoral activation of a marker gene in transformed plants was by Odell *et al.* (1994). These authors placed Cre under the control of seed-specific promoters, and used tissue-specific Cre expression to activate GUS from a construct similar in design to the target construct described here. In certain tobacco lines with low levels of Cre expression, GUS activity was induced in sectors of tissue. This work differs in two important respects to that reported here. First, the timing of recombinase activity and hence marker gene activation could not be controlled. Second, the activation event placed GUS under the same seed-specific promoter that was used to express Cre, limiting subsequent GUS expression to cells that express this promoter. In contrast, our activation event placed GUS under the control of the CaMV 35S promoter, which is known to be expressed in a wide variety of cell types and developmental stages. For example, the 35S CaMV promoter appears to be able to direct GUS gene expression in all vegetative structures of *Arabidopsis* (Figure 7c). Thus, at least in the case of *Arabidopsis*, we believe that our approach marks irreversibly all cells derived from an activation event.

Although the use of site-specific recombinases for activating gene expression by excision of a transcription blocking sequence resident between tandemly repeated target sites in transgenic plants has been previously reported using Cre (Bayley *et al.*, 1992; Dale and Ow, 1991; Odell *et al.*, 1994) and FLP (Lloyd and Davis, 1994), to our knowledge all such experiments involving gene activation have been carried out in tobacco; the work of Russell *et al.* (1992) in *Arabidopsis* (cited in the introduction of this paper) involved following only the loss of a transgene. In contrast to the work of Lloyd and Davis (1994), we have been successful in observing FLP activity in transgenic *Arabidopsis* plants. We believe that this difference may be a result of using an inducible heat-shock promoter since, to date, we have also been unsuccesful in obtaining *Arabidopsis* transformants with constitutive FLP activity (data not shown).

The convenient methodology for inducing marked cell clones that is presented in this paper offers a number of possibilities for more detailed analysis of cell fate, including

cell lineage analysis in mutants that have altered development. The ability to generate clonal sectors in which specific genes have been activated or removed not only permits clonal genealogies to be followed, but also provides a powerful analysis system for investigating the function of genes likely to affect cell physiology or developmental fate. Elevated expression from heterologous promoters or reduced expression by antisense regulation of such genes can be informative about their biological function, but the constructs required may be difficult to introduce into plants, since their activity may affect the transformation process. We are currently modifying the system presented here, to allow such genes to be introduced into plants in an inactive state, and subsequently activated in sectors of cells by heat-shock-induced expression of FLP recombinase. These sectors can be labelled by the concomitant removal of a marker gene with a visible phenotype. The expression level of this marker gene before FLP excision would also reflect the likely expression level of the gene of interest after activation.

Furthermore, heat-shock-induced FLP activity can be used for a strategy of 'clonal gene knockout' in situations where a mutant plant and corresponding cloned gene are available. In this situation, a wild-type allele of the cloned gene flanked by FRTs is transformed into a wild-type plant. Such plants are crossed to the mutant, and plants identified in which the only active copy of the gene is the transgenic flanked by FRTs. This can be subsequently removed in clones of cells, whose developmental fate can be compared with adjacent wild-type tissue by incorporating appropriate markers.

Experimental procedures

Plasmid constructions

FLP recognition targets (FRTs). Two FRTs (designated FRT1 and FRT2) containing both the minimal FLP recognition sequence and the additional flanking repeat (Kilby *et al.*, 1993) were synthesized as self-complementary oligonucleotides and annealed. FRT1 and FRT2 differ only in the flanking restriction sites introduced to facilitate cloning. Forward (5'–3') oligonucleotide sequences were as follows:

*Hin*dIII–**FRT1**–*Bam*HI: AAG CTT GCT TTG AAG TTC CTA TTC CGA AGT TCC TAT TCT CTA GAA AGT ATA GGA ACT TCA GGA TCC .

*Bgl*II-**FRT2**–*Hin*dIII: AGA TCT GCT TTG AAG TTC CTA TTC CGA AGT TCC TAT TCT CTA GAA AGT ATA GGA ACT TCA GAA GCT T.

*Hin*dIII–**FRT1**–*Bam*HI was cloned between the *Hin*dIII and *Bam*HI polylinker sites of pEMBL131$^+$ (Cesareni and Murray, 1987), creating pEMBL131$^+$ FRT1 (pMS9; Snaith, Kilby and Murray, submitted). *Bgl*II–**FRT2**–*Hin*dIII was cloned between the *Bam*HI and *Hin*dIII polylinker sites of pEMBL131$^+$ by *Bgl*II/*Bam*HI and *Hin*dIII/*Hin*dIII cohesive end ligation, creating pEMBL131$^+$ FRT2 (pMS10; Snaith, Murray and Boulter, submitted).

FLP (excision) target construct. The FLP target construct pNJK14 is shown in schematic form in Figure 2(a) and construction details are given in Figure 8. Cloning of pNJK14 was as follows: pEMBL131$^+$ FRT2 (pMS10) was digested with *Pst*I and *Sst*I. The FRT2-containing fragment from this digest was cloned into the *Pst*I and *Sst*I sites of pBluescript^{TM} II SK$^+$ (Stratagene, La Jolla, CA) creating pFRT2 Bluescript (pNJK10). pNJK10 was then digested with *Eco*RI and *Sst*I. The FRT2-containing fragment liberated by this digest was then cloned into the *Eco*RI and *Sst*I sites of pNJK09 (pUC18 (Pharmacia Biotech, Brussels, Belgium) previously cut with *Sph*I and *Xba*I, blunted with Klenow fragment, and religated) to create pUC18 FRT2 (pNJK11). In parallel, pJC3 (Lichtenstein, personal communication) was cut with *Xba*I and *Bam*HI to yield a 2.2 kb fragment containing the hygromycin coding region and octopine synthase 3' transcription termination sequences. This 2.2 kb fragment was blunted with Klenow fragment and ligated between the *Hin*dIII site (previously blunted with Klenow fragment) and EcoRV site of pEMBL131$^+$ FRT1(pMS9) as a (*Hin*dIII/*Xba*I) and (*Bam*HI/*Eco*RV) blunt end ligation, creating pEMBL131$^+$ FRT1 HYG ocs (pNJK08). pNJK08 was digested with *Bam*HI and *Sst*I and the resulting FRT1 HYG ocs-containing fragment then cloned into the *Bam*HI and *Sst*I sites of pNJK11 to create pUC18 FRT1 HYG ocs FRT2 (pNJK13). pNJK13 was digested with *Sal*I and *Bam*HI and the resultant FRT1 HYG ocs FRT2-containing fragment then blunted with Klenow fragment and cloned into the Klenow-blunted *Xba*I site of pNJK12 to produce the FLP (excision) target construct pNJK14; pNJK12 was constructed by cloning the 35S CaMV promoter–GUS–nos cassette from pC-TAK1 (Kavanagh, personal communication) as a *Hin*dIII/*Eco*RI fragment into the *Hin*dIII and *Eco*RI sites of pGA482 (An, 1986). The essential feature of pNJK14 is that it contains a 35S CaMV promoter and GUS coding region, interrupted by a FLP-excisable hygromycin reistance cassette (35S FRT1 HYG ocs FRT2 GUS nos) located between T-DNA borders.

FLP (excision) target: positive control construct. The recombinant, excision-product produced by FLP-mediated recombination between the FRT sites of pNJK14 was obtained by *in vivo* FLP-mediated recombination. This was achieved by transforming pNJK14 into *Escherichia coli* BL-FLP, a strain which expresses FLP recombinase (Snaith, Kilby, and Murray, submitted). This positive control construct was designated pNJK15 (Figure 2b).

Constitutive FLP source. A constitutive source of FLP recombinase, designated here as 35S Δ FLP (Figure 3a) was constructed as follows. Plasmid pY39 (pUC19 containing the *Sph*I/*Xba*I fragment of 2 μm plasmid from *Saccharomyces cerevisiae* encompassing the native FLP coding region) was digested with *Hga*I (*Hga*I cuts at the 3' end of the FLP gene; Hartley and Donelson, 1980), blunted with Klenow fragment, then cut with *Hin*dIII, and the 3' portion of the FLP coding region isolated. In parallel, the 5' portion of the FLP gene was also isolated from pY39 as an *Sph*I/*Hin*dIII fragment. Both these fragments were then cloned into the *Sph*I and *Eco*RV sites of pEMBL131$^+$ as a three way ligation to produce pEMBL131$^+$ FLP (pMS11). pEMBL131$^+$ FLP (pMS11) was digested with *Hin*dIII to remove the 5' end of the native FLP coding region which was replaced with the *Hin*dIII/*Hin*dIII fragment of the FLP coding region from pOG44 (O'Gorman *et al.*, 1991), creating pEMBL131$^+$ΔFLP (pMS14). pOG44 contains a modified FLP gene in which three canonical AATAAA polyadenylation signals have been removed from the 5' portion of the coding region without altering the amino acids encoded. pEMBL131$^+$ΔFLP (pMS14) was

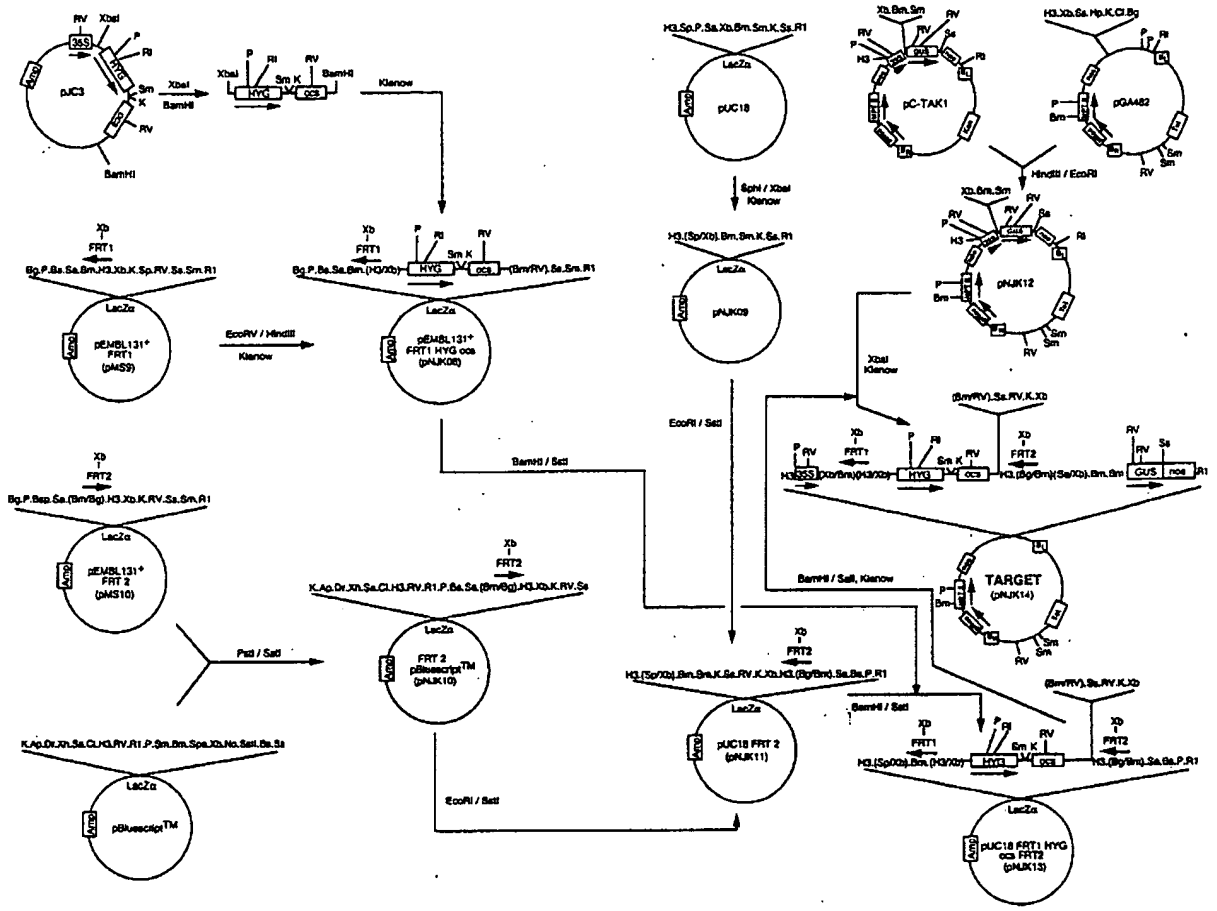

Figure 8. Construction of the FLP target construct, pNJK14.
Ap, *Apa*I; Bg, *Bgl*II; Bm, *Bam*HI; Bs, *Bst*XI; Bsp, *Bsp*MI; Cl, *Cla*I; Dr, *Dra*II; H3, *Hin*dIII; K, *Kpn*I; No, *Not*I; P, *Pst*I; R1, *Eco*RI; RV, *Eco*RV; Sa, *Sal*I; Xb, *Xba*I; Sm, *Sma*I; Sp, *Sph*I; Spe, *Spe*I; Ss, *Sst*I; SsII, *Sst*II; Xh, *Xho*I; bracketed restriction sites represent blunt-end ligations in which cohesive-end restriction sites were rendered flush by treatment with Klenow fragment of DNA polymerase I. Amp, ampicillin resistance marker; Tet, tetracyclin resistance marker; B_L, left T-DNA border; B_R, right T-DNA border; FRT= FLP recognition target; GUS, b-glucuronidase coding region; HYG, hygromycin coding region; Kan, kanamycin resistance cassette; pNos, nopaline synthase promoter; nos, 3' transcription termination signals fronm the nopaline synthase gene; NPT II, neomycin phosphotransferase II coding region; Neo, neomycin phosphotransferase resistance cassette; ocs, 3' transcription termination signals from the octopine synthase gene; 35S, 35S cauliflower mosaic virus promoter; Tet, tetracyclin resistance cassette.

restricted with *Bam*HI and *Sst*I to remove the chimeric ΔFLP coding region, which was blunted with Klenow fragment and cloned into the Klenow-blunted *Xba*I/*Sst*I sites of pNJK12; the transcriptionally correct (5'–3') insert orientation was confirmed by restriction analysis. This procedure placed the ΔFLP coding region in a binary vector under the control of a 35S CaMV promoter with nos 3' transcription termination sequences.

Inducible (heat-shock) FLP source. An inducible source of FLP recombinase, designated HSP FLP (Figure 3b) was constructed using the native FLP recombinase coding region. pEMBL131$^+$FLP (pMS11) was digested with *Xba*I and *Sst*I to excise the FLP coding region which was then blunted with Klenow fragment and cloned into the Klenow-blunted *Bam*HI site of pHSLEX (Schöffl; personal communication), a pBIN19 (Bevan, 1984) derivative; the transcriptionally correct (5' to 3') insert orientation was confirmed by restriction analysis. This placed the FLP coding region in a binary vector under the control of the *Gmhsp* 17.6L soybean heat-shock promoter (Severin and Schöffl, 1990) with nos 3' transcription termination sequences.

All FLP expression constructs, the FLP target and the positive control vector were mobilized into *Agrobacterium tumefaciens* strain C58C1^{rif} by triparental mating (Ditta *et al.*, 1980), in preparation for plant transformation.

Plant transformation

Agrobacterium-mediated transformation of *Arabidopsis thaliana* Landsberg *erecta* and Columbia ecotypes was essentially as described (Valvekens *et al.*, 1988), except that shoot induction medium was modified to include 0.4 mg l^{-1} *N*-6-benzylaminopurine and 0.2 mg l^{-1} napthalene acetic acid; other plant growth regulators being omitted. Shoot induction, root induction and seed germination were performed using DMS medium. DMS is MS medium (Murashige and Skoog, 1962), containing sucrose (1%w/v), agar

(0.8%w/v; pH5.7). Transformed microcalli were generated by incubating co-cultivated root explants for 2 weeks on callus induction medium (DMS medium + 0.5 mg l^{-1} 2,4-dichlorophenoxy acetic acid and 0.05 mg l^{-1} kinetin) containing 750 mg l^{-1} vancomycin hydrochloride (Eli Lilly, UK) and 50 mg l^{-1} kanamycin sulphate. Shoots were rooted by incubating explants on DMS medium containing 1mg/l indole-3-butyric acid. Rooted plants were transferred to soil to set seed. Seeds from transgenic plants were surface-sterilized and germinated on DMS medium containing 50 mg l^{-1} kanamycin sulphate. Seedlings were scored as resistant (green cotyledons) or sensitive (yellow cotyledons) after 2 weeks growth on kanamycin-containing germination medium.

Agrobacterium-mediated transformation of *Nicotiana tabacum* var. xanthi leaf discs was as described (Horsch *et al.*, 1985). For scoring of antibiotic resistance, vernalized (5 days at 4°C), surface-sterilized seeds from transgenic plants were placed on DMS medium containing 100 mg l^{-1} kanamycin sulphate, set in vertically mounted, square 'Sterilin' dishes (Bibby Sterilin Ltd, UK). Seedlings were scored as resistant (long root) or sensitive (short root) after 2 weeks growth. The average root length of wild-type seedlings grown under these conditions was five-fold less than that of transformed, kanamycin-resistant seedlings or of wild-type seedlings grown without selection.

Plant DNA extraction and analysis

Total genomic DNA was prepared from axenically grown or soil-grown plants, as appropriate. DNA extraction and purification were as described (Murray and Thompson, 1980), except for extraction of nucleic acids from tissues destined for PCR analysis during clonal sector studies. Here, seedlings were frozen in liquid nitrogen, ground to a fine powder and then solubilized in extraction buffer (10 mM Tris, 1 mM EDTA, pH 8). The resultant suspension was centrifuged to remove cell debris and total nucleic acids in the supernatant were ethanol prepitated at –70°C using 1/10 volume 5 M NaCl. Precipitated nucleic acids were dissolved in extraction buffer (1 ml) prior to PCR analysis (10 ml of sample per reaction volume). Restriction digests of *Arabidopsis* DNA (2 µg) and tobacco DNA (10 µg) were performed with a 10-fold excess of enzyme in the presence of 0.4 µM spermidine. Southern blots were produced using Genescreen Plus nylon transfer membrane (Dupont NEN Research Products, Bloomington, IN) according to the manufacturer's instructions.

Plant RNA extraction and analysis

Total RNA from axenically grown or soil-grown plants was isolated as described (Soni and Murray, 1994), except that RNA was selectively precipitated from the aqueous phase using 2 M LiCl. For transcript analysis, total RNA (30 µg per sample) was separated in a 1.5% (w/v) agarose (Boehringer Mannheim GmbH, Germany) gel containing 16% formaldehyde then transferred to Genescreen Plus nylon transfer membrane (Dupont NEN Research Products, Bloomington, IN) according to the manufacturer's instructions. All DNA probes were radio labelled with [α-^{32}P]dATP by random priming (Feinberg and Vogelstein, 1983,1984).

Histochemical localization of GUS activity

Gus activity in plant tissues was localized by histochemical staining with 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc, Biosynth AG, Staad, Switzerland) as described (Jefferson, 1987). Tissues were incubated in X-Gluc containing reaction buffer at 37°C overnight, then destained in 70% ethanol prior to photography.

Heat-shocking of plants

Seeds and germinating seedlings maintained on DMS medium in 'Parafilm' sealed plastic petri dishes were heat-shocked in a microbiological incubator, at 37°C for various times (see Results). Whole plants in soil were similarly heat-shocked in enclosed plant propagators containing a reservoir of water to maintain humidity.

PCR analysis

Total genomic DNA (300 ng) was used as substrate for PCR amplification. Each reaction mix (total reaction volume of 100 µl overlayed with 75 µl of mineral oil) contained: 0.25 mM of each primer; 0.1 mM dNTPs; buffer (100 mM Tris, pH 8.5, 15 mM NH_4Cl, 500 mM KCl); 1 mM $MgCl_2$; 1 unit of BioTaq™ polymerase (Bioline U.K. Ltd, London). PCR amplification was carried out using a 'LEP Scientific' thermal cycler (PREM™) run for 30 cycles. The PCR reaction conditions used (Lloyd and Davis, 1994) were as follows: denaturation at 94°C, 4 min; primer annealing at 55°C, 30 sec; elongation by BioTaq™ polymerase at 72°C, 45 sec; denaturation at 94°C, 30 sec; and a final extension step at 72°C, 7 min. PCR products were separated in a 1% (w/v) agarose gel then visualized by staining with ethidium bromide. The forward oligonucleotide sequence (P1) used to prime from the 3' end of the 35S CaMV promoter was as follows: 5'–GTG GAT TGA TGT GAT ATC TCC–3'. The reverse oligo nucleotide sequence (P2) used to prime from the 5' end of the GUS coding region was as follows: 5'–ATC TGC ATC GGC GAA CTG ATC G–3'.

Restriction analysis of PCR excision product

PCR excision product was electro-eluted from a 1% agarose gel, ethanol-precipitated and re-suspended in 10 mM Tris–HCl, 1 mM EDTA, pH 8. Gel-purified PCR excision product was then restricted with *Xba*I and electrophoresed on a 4% NuSieve® GTG® (FMC BioProducts, Rockland, ME, U.S.) agarose gel to resolve restriction products. Undigested, gel-purified PCR product was electrophoresed on the same gel for comparison.

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